

Cu and Zn relations in fertilised eucalypt plantations

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Declarations

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgment is made in the text of the thesis.



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Abstract

Interactions between plant nutrients are common and high levels of one nutrient are often associated with a reduced concentration of a second nutrient. For example, an increased growth rate of the plant due to the application of one nutrient can lead to dilution and may cause deficiency of that second nutrient. Effects of one nutrient on root system architecture and mycorrhizal infection may interfere with the uptake of other nutrients. Specific interactions between nutrients have also been reported (Robson & Pitman, 1983). These may lead to increased adsorption to soil solids and changes in the rate of diffusion in soil, interference in uptake and transport by competition, and the formation of insoluble complexes within the plant. A reduction of Cu and Zn concentrations due to application of high levels of N and P has been reported for a number of annual crops and in this thesis it will be investigated for *Eucalyptus nitens*.

Australia aims to treble its plantation estate by 2020 and new plantations have been established recently at a rate of 80,000 ha per annum. As a result of policies directed towards the conservation of native forest, land previously used for agriculture is increasingly used for the establishment of plantations. Poor stem form of trees grown on ex-agricultural land is a common problem particularly in combination with high rates of applied N and P. This poor stem form has been linked with Cu deficiency in *Pinus radiata* (Ruiter, 1969) and *Eucalyptus nitens* (Turnbull *et al.*, 1994). A preliminary investigation of research data and the field site used by Turnbull *et al.* (1994) indicated that Zn deficiency and high levels of Mn may have interacted with

Cu deficiency to cause the stem deformities. This thesis investigates the interaction of high N and P application with Cu, Mn and Zn nutrition of *E. nitens*.

A series of greenhouse trials were conducted using soil from a site at which stem deformities have occurred. The first experiment investigated the interaction between N, P, Cu, Mn and Zn application on the growth and micronutrient concentration of *E. nitens* seedlings grown in soil associated with straight or deformed trees in the field. *Lycopersicon esculentum* was investigated as a test plant for micronutrient disorders in this experiment. In a second the effect of Cu application on Cu concentrations in *L. esculentum* and *E. nitens* seedlings on two soils was tested. In the third experiment the effect of Cu, Mn and Zn application on growth, stem form and micronutrient concentration of *E. nitens* saplings grown at high levels of N and P was investigated. The fourth experiment investigated the effect of N and Cu application on the uptake and distribution of Cu by *E. nitens* clones.

Soil that was associated with stem deformities in the field resulted in a reduced uptake of Cu and Zn and a lower growth response to the application of N and P by *E. nitens* and *L. esculentum* than soil that was associated with straight trees in the field. When Cu and Zn were applied there was an increase in tissue concentration of the respective nutrient. *L. esculentum* proved to be a good indicator plant for Cu deficiency in *E. nitens* seedlings. The Cu concentration in *L. esculentum* was in the deficiency range when compared to that reported previously in the literature, while Zn concentrations were adequate. The growth response to the application of micronutrients was small. The largest response occurred with Cu application in combination with high N and P application.

High N application reduced the Cu concentration in seedlings grown without Cu application. With applied Cu, high N application resulted in increased Cu concentration. N application improved the root to shoot transport of applied Cu. The root to shoot transport of Cu was subject to genetic variability with one clone of *E. nitens* retaining a significantly higher proportion of Cu in the roots than the other.

Stem deformities like those observed in the field could not be induced during the greenhouse experiments, although Cu concentrations were deficient for growth. This may be due to the short duration of the greenhouse experiments (up to 6 months) as the symptoms in the field occurred only in the second growing season. Another possible explanation may be the absence of wind stress and other triggers of stem deformities in the controlled greenhouse environment.

The research in thesis showed that N application was the cause of the induced Cu deficiency observed in the greenhouse. It showed that the effect of N application on the foliar Cu concentration occurred during the root to shoot transport. The deficiency was ameliorated with Cu application and seedlings with high N and P application showed increased growth to applied Cu. The research supported the critical Cu concentration of 1.4 mg/kg proposed by Turnbull *et al.* (1994), which had previously been based on a single experiment and showed that it is likely to also indicate a growth response to Cu application at high N and P. Cu concentration of *L. esculentum* was a good indicator of Cu deficiency in *E. nitens* seedlings in the greenhouse.

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Chapter 1: Introduction

1.1 Background

The growing of eucalypts in plantations for the production of sawlogs and pulpwood is becoming increasingly important in Australia. The area of eucalypt plantations in Tasmania increased from 48,000 ha in 1993 to 73,000 ha in 1998 and is currently around 120,000 ha. It was planned to establish 20,000 ha per annum of plantations in Tasmania until 2003 (Anon, 1998). Nationally, the Australian federal government aims to treble the plantation estate in Australia by 2020 (Anon, 1999). To reach this target, it will be necessary to establish new plantations at an average rate of 80,000 ha per annum. The establishment of plantations on land previously used for agriculture is a consequence of policies directed towards conservation of native forests.

Trees growing on ex-agricultural land often show problems with stem form. This poor form resembles the expression of symptoms arising from Cu deficiency and trees often have low Cu concentrations. It has been described for *Pinus radiata* in Australia (Birk, 1990; Ruiter 1969) and New Zealand (Hunter et al., 1990). It is possible that in some cases the deformities are a result of an N-induced Cu deficiency as a high nitrification rate has been identified as one of the contributing factors (Carlyle *etal.*, 1989).

Poor stem form due to suspected micronutrient deficiencies induced by high N and P application have appeared in eucalypt plantations. This was first described in *E. nitens* by Turnbull et al. (1994) and occurred in a fertiliser trial at Gould's Block near Dover, Tasmania. Trees receiving a high rate of N and/or P showed severe deformities, while

the unfertilised controls showed straight growth (Turnbull *et al.*, 1994). Cu deficiency was again a possible cause. Turnbull *et al.* (1994) reported a significant relation between Cu levels in the leaves and the degree of poor stem form. Severe deformities of the 21-month-old saplings occurred at foliar Cu concentrations below 1.4 mg/kg. Poor stem form has since been observed in a number of ongoing fertiliser trials with *E. nitens* and *Eucalyptus globulus* (unpublished data).

The degree of deformity associated with poor stem form ranges from single kinks in the stem to sinuous growth of the whole stem. In very severe cases trees are multi-leadered with severely twisted leaders and branches. Although the growth rate of the trees is only reduced in the more severely deformed trees, poor tree form results in a loss of marketable timber. Turnbull *et al.* (1994) found that the percentage of prunable trees (used for veneer and sawlog production) was reduced by three quarters due to the effect of N and P application on tree form. Poor stem form will also increase the effort and cost of handling and cartage, which will almost certainly make harvesting of severely deformed trees uneconomic. No numbers are available as yet on the impact of poor stem form on harvestable yield from eucalypt plantations, but experience with *P. radiata* shows that potential for large losses exists. Birk (1990) reported the potential loss from a 150 ha plantation as a 27% reduction in expected yield and for more than 30% of the harvest a reduction in price of \$2 per tonne due to defects.

The research of Turnbull *et al.* (1994) did not investigate the interaction between the high N and P application in their trial and the Cu concentration of the *E. nitens* saplings. In general, little is known about the effect of N and P application on nutrition

with micronutrients in *E. nitens*. On the other hand, there have been many studies of this effect for a number of important crop plants and many interactions have been identified. Increased growth rate of the plant due to the application of one nutrient can lead to the dilution of a second and may cause deficiency of that nutrient. There are also specific interactions which lead to increased adsorption and changes in the rate of diffusion in soil, effects on root system architecture and mycorrhizal infection, interference in uptake and transport by ion competition, and the formation of insoluble complexes within the plant (Robson & Pitman, 1983). A reduction of Cu and Zn concentrations due to application of high levels of N and P has been reported for a number of annual crops and in this thesis it will be investigated for *E. nitens*.

1.2 Objectives of the thesis

The research in this thesis investigates the uptake and distribution of Cu and Zn by *E. nitens* seedlings at high rates of N and P application. Stem deformities associated with fertiliser-induced Cu deficiency in *E. nitens* that were first reported by Turnbull *et al* (1994) were also investigated. A preliminary investigation of research data and the field site used by Turnbull *et al.* (1994) indicated that Zn deficiency and high levels of Mn may have interacted with Cu to influence tissue levels in *E. nitens* and exacerbate the expression of symptoms. It was also found that the expression of symptoms was related to soil variability at the site and the genetic variability of trees.

The research in this thesis was specifically directed towards to the following questions in relation to nutrition of *E. nitens*.

- 1) Investigate interactions between N and P fertilisation and uptake of Cu and/or other micronutrients.
- 2) Identify soil and plant factors involved in the interaction between N and P fertilisation and micronutrient nutrition.
- 3) Were deficiencies of Cu and/or other micronutrients connected with the stem deformities reported by Turnbull *et al.* (1994)?

1.3 Structure of the thesis

An outline of chapters and their content is given to show the development of the research programme.

Chapter 1: Introduction

This has already been dealt with and describes the background to the research undertaken and the objectives and structure of the thesis.

Chapter 2: Plant nutrition with Cu and Zn in plants fertilised with N and P

This chapter reviews the current knowledge of the soil supply and the plant uptake of Cu and Zn and their interaction with high rates of N and P. It also provides an overview of methods used to detect deficiencies of Cu and Zn and to conduct research with these micronutrients.

Chapter 3: Cu deficiency and stem deformities in fertilised plantation eucalypts – a field study

This chapter contains a reanalysis of data previously obtained by Charles Turnbull and others and the results of a preliminary field study. Data showing the effect of nutrient concentration on stem deformities, the effect of N and P application on nutrient concentrations and the effect of application of micronutrients in an amelioration trial are presented. During the field study several trees showing differing severity of stem deformities were harvested, including excavation of the root systems. Roots and soil samples from the root zone were taken from further pairs of straight and severely deformed trees. Plant material and soil were analysed for Cu, Mn and Zn concentrations.

The research in this chapter attempts to show that high N and P application resulted in reduced concentrations of Cu and to identify other nutrients that may be associated with the stem deformities in the field. It was shown that N and P applications reduced foliar Cu concentrations in plantation-grown *E. nitens* saplings. Stem deformities were not only associated with Cu deficiency, but also with lower Zn concentration and an increase of Mn concentration in roots. Application of Cu was effective in raising foliar Cu concentration and ameliorating stem deformities. Differences in the root systems of straight and deformed trees, and the soil they grow on are described.

Chapter 4: Fertiliser-induced micronutrient deficiency in *E. nitens*: greenhouse experiment using seedlings, clones and a test plant

The research in Chapter 3 identified Zn deficiency and Mn toxicity as possible factors interacting with Cu deficiency as the cause of stem deformities. Differences in soil under deformed and straight trees were also identified. Chapter 4 describes two greenhouse experiments investigating the interaction of N and P with Cu, Mn and Zn nutrition of *E. nitens* and the suitability of *L. esculentum* as a test plant for *E. nitens*. The first experiment is a factorial experiment with N, P, Cu, Mn and Zn application and soil associated with straight and deformed trees in the field as factors. The second experiment uses a different level of Cu application and an additional soil from a site where stem deformities have occurred in *Eucalyptus globulus*.

The research in the first experiment of this chapter attempts to show that high N or P application will reduce Cu and/or Zn concentration in *E. nitens* seedlings. Based on the research of the previous chapter the first experiment aims to identify if Zn deficiency and Mn toxicity plays a role in the interaction between Cu nutrition and N and P application on the growth of the seedlings. The difference between soils associated with straight and deformed trees on the interaction between the nutrients is also explored. It is attempted to indicate Cu and Zn deficiency in *E. nitens* by the use of *L. esculentum* as a test plant in both experiments.

Chapter 5: Effect of Cu, Mn and Zn application on the growth, stem form and micronutrient concentrations of *E. nitens* seedlings

This chapter describes a greenhouse experiment which further investigates the interaction of Cu, Mn and Zn nutrition on growth and nutrient concentration of *E. nitens*. The growth response to the interaction Cu, Mn and Zn application in Chapter 3 did not give clear results. Additional rates of nutrient application were therefore used and larger pots allowed the seedlings to be grown for a longer time than in the previous experiments.

Chapter 6: Effect of N and Cu nutrition on the distribution of Cu in *E. nitens* clones

The results of Chapter 4 showed that N application resulted in reduced Cu concentration in *E. nitens* seedlings. Chapter 6 describes a greenhouse experiment investigating the effect of two rates of N application and four rates of Cu application on two *E. nitens* clones. The effect of N and Cu application on the transport and remobilisation of Cu has been well studied in *Triticum aestivum* (Hill *et al.* 1978, 1979 a,b; Loneragan *et al.*, 1980). Based on the results of those experiments the research in this chapter attempts to show that the application of N will inhibit senescence of leaves and the remobilisation of Cu from senescent leaves.

The effect of N and Cu application on the shoot : root ratio and the distribution of Cu between roots and shoots of *E. nitens* seedlings was also investigated. The aim was to show that increase in the shoot : root ratio with high N application reported previously for many plant species (Wilson, 1988) and retention of Cu in the roots contributed to Cu deficiency in the shoots of the seedlings. Two clones of *E. nitens* are used in this

experiment in an attempt to identify differences of Cu nutrition in plants with genetically different background.

Chapter 7: General Discussion

This chapter presents an analysis of key outcomes from all experimental chapters relating to the induction of Cu and Zn deficiency by high N and P application. The uptake and distribution of Cu and Zn in the soil-root-shoot system and the effect of nutrient application on the growth and shoot : root ratio of *E. nitens* are discussed. Implications of the research for the diagnosis and management of Cu deficiency in *E. nitens* including the use of *L. esculentum* as a test plant are presented as well.

Chapter 2: Cu and Zn nutrition in plants fertilised with N and P

2.1 Soil supply of Cu and Zn

2.1.1 Natural occurrence of Cu and Zn

The average concentration of Cu in the lithosphere is 70 mg/kg with higher concentrations in basaltic rock compared to granitic rock (McBride, 1981). The total Cu concentration of soils ranges from trace levels to 250 mg/kg: most soils contain between 15 and 40 mg/kg total Cu (Aubert & Pinta, 1977). Cu can be found naturally as the native metal. More often it is present as sulfide minerals, both alone (eg. chalcocite - Cu_2S) and in mixed metal minerals (eg. chalcopyrite - CuFeS_2). Oxidation of sulphides results in a number of minor minerals containing Cu(II). During weathering of primary minerals, Cu(II) can be held in the octahedral position in layer silicates due to isomorphous substitution (McBride, 1981).

Zinc is considered the 25th most abundant element and its average concentration in the upper continental crust has been estimated to be 57 mg/kg. The total Zn concentration of soils varies from trace levels to 900 mg/kg with an average concentration between 50 and 100 mg/kg (Aubert & Pinta, 1977). The major ores of commercial interest are a sulfide (sphalerite - ZnS), carbonate (smithsonite - ZnCO_3) and a silicate (hemimorphite - $\text{Zn}_4\text{Si}_2\text{O}_7(\text{OH})_2\cdot\text{H}_2\text{O}$). Zinc is also found as a minor constituent in other minerals after isomorphic substitution (Barak & Helmke, 1993).

2.1.2 Soil speciation of Cu and Zn

The distribution of Cu and Zn in different soil fractions in eight soils representative of those in the south-eastern United States was studied by Shuman (1979). He improved the fractionation scheme in a later study (1985) that included 16 soils. There are also other soil fractionation studies into the distribution Cu (McLaren & Crawford, 1973a; Flores-vélez *et al.*, 1996; Rupa *et al.*, 2001) and Zn (Iyengar *et al.*, 1981). All schemes have a common feature in that they sequentially extract the materials with extractants that dissolve a certain fraction of the element. Common fractions are: solution, exchangeable, organically bound, bound to amorphous and crystalline Fe, Al and Mn oxides, and a residual fraction. The methods used for the fractionation of Cu and Zn in soils are similar to those used for trace element analysis of other environmental materials such as particulate matter in waters (Tessier *et al.*, 1979). The major limitation of these schemes is whether the extractant truly represents the fraction studied (Flores-vélez, 1996; Shuman, 1985). To test the validity of the extraction schemes, the total element concentration and the sum of the fractions were compared. Good correlation between the proportion of metal contained in soil fractions and the relative size of the respective soil fraction was also taken as evidence of good representation by the extraction scheme (Flores- vélez, 1996; McLaren & Crawford, 1973 a).

The earliest systematic attempt to fractionate soil Cu was that of McLaren and Crawford (1973a). They analysed 24 British soils for Cu contained in five soil fractions. They considered soil solution plus exchangeable Cu (CaCl_2), specifically absorbed Cu (acetic acid), organically chelated Cu (pyrophosphate), Cu occluded by free iron and manganese oxides (acid oxalate) and residual soil Cu (hydrofluoric acid).

Their choice of extractants was based on theoretical assumptions about the source of the Cu extracted. Their results showed that the total Cu content of their soils varied considerably between the lowest and the highest concentration (4.4-63.5 mg/kg). Good correlation between the total Cu content of the soils and the major fractions of Cu in the soil led them to suggest that for comparative purposes the concentration should be expressed relative to the total Cu content. Exchangeable and specifically adsorbed Cu was only a small proportion of total Cu. Only in one soil did they make up 5% of total Cu while in all others they contributed less than 2%. On average 50% of Cu remained in the residual fraction, while 30 and 15% were associated with organic matter and metal oxides respectively. The average total Cu content of soils studied in the US (Shuman, 1979, 1985) was lower than that found in Great Britain, but of a similar range (1.6 – 47.3 mg/kg). Shuman (1979) found a similar distribution of Cu in the soil fractions, but a slightly higher proportion in organic matter and less in the metal oxides. The difference is likely caused by the use of peroxide for the extraction of organic matter as this would have resulted in the dissolution of some MnO₂. In the later study (Shuman, 1985), most of the Cu was in the residual and the Fe-oxide fraction, while the organic matter fraction made up only 10-15%. The differences in the results can be linked again to the extractant used for the organic matter, as he used NaOCl which does not dissolve MnO₂. Flores-vélez *et al.* (1996) reported similar percentages in organic matter using the same method (NaOCl). The discrepancies between the above studies show the problems in relating actual soil fractions to those extracted by reagents.

The proportion of Zn found in the organic matter fraction was lower than that of Cu, while a larger amount was contained in the residual fraction (Iyengar *et al.*, 1981;

Shuman, 1979, 1985). Iyengar *et al.* (1981) found that a large proportion of Zn (up to 14 %) was specifically adsorbed. A large proportion of the residual Zn was associated with the clay fraction even in sandy soils and in soils with a higher clay content almost half the total Zn was contained in the residual clay fraction (Shuman, 1985)

2.2 Plant uptake of Cu and Zn

Plants take up the majority of their nutrients from the soil via the root (Jungk & Claasen, 1997). Foliar uptake is not considered in this review, though micronutrients are often applied by foliar application of liquid fertiliser. Rain is also a potential source for foliar uptake of micronutrients, particularly near industrial centres or other sources of atmospheric pollution (Hewitt, 1966).

2.2.1 Soil as a system of solids and soil solution

Soil is a highly heterogeneous system of solid particles and pores (Jungk & Claasen, 1997). The pores are filled with water to a varying degree. This depends on the soil water content and the pore diameter. The smaller pores are filled with water and the remainder of the pores contain the soil atmosphere. When considering uptake of nutrients from the soil, the contribution of the soil atmosphere is negligible for non N-fixing plants, provided that the soil is aerated. Sufficient O₂ has to be present in the soil to allow active uptake processes to be driven by root respiration. In the case of wetland plants the O₂ may be supplied from the atmosphere through specialised root tissue.

Plants take up the nutrients from the soil solution and the uptake rate therefore depends on the solution concentration of the nutrient. The concentration of the nutrient in the soil solution is buffered by its dynamic equilibrium with the soil solids. The interface between the solids and the liquid is made up of several layers (Figure 2.1). The pools of nutrients contained in these layers are of different importance for different nutrients. Cations that form a strong hydration shell are mainly held in the cation exchange complex (CEC). The CEC occurs because of the negative charge on the soil solids. Cations are attracted electrostatically to this charge and the establishment of an electrochemical equilibrium results in a higher concentration of cations near the solid surface than in the bulk soil solution. The currently accepted model is the double-layer boundary: this model has been reviewed extensively by Attard (1996). The ions held in the CEC are readily exchanged against other ions present in the soil solution. The degree by which ions are attracted to the double layer increases with increasing charge of the ion. The extent of the CEC depends largely on the surface area of the particles and their charge per unit surface area. Colloidal clay and soil organic matter (SOM) are the main contributors to the CEC because of their high effective surface area due to the small particle size. Particles acquire charge in a permanent or pH-dependent way. Permanent charge occurs in some clay minerals and results from the isomorphic substitution of lower valency ions in the crystal lattice of clay minerals (Bolan *et al.*, 1999). This leaves some of the negative charge of the O atoms unneutralised. A pH-dependent charge occurs when O atoms at the edge of crystals with free electron pairs and nucleophilic functional groups in the SOM have a negative charge. The negative charge on these materials depends on the protonation of the electron pairs and is reduced by a lower soil pH. The charge at the surface of solids is also changed by specific cation and anion adsorption (Bolan *et al.*, 1999).

Cation exchange complex in a diffuse double layer resulting from the Donnan equilibrium with negative charges at the solid surface

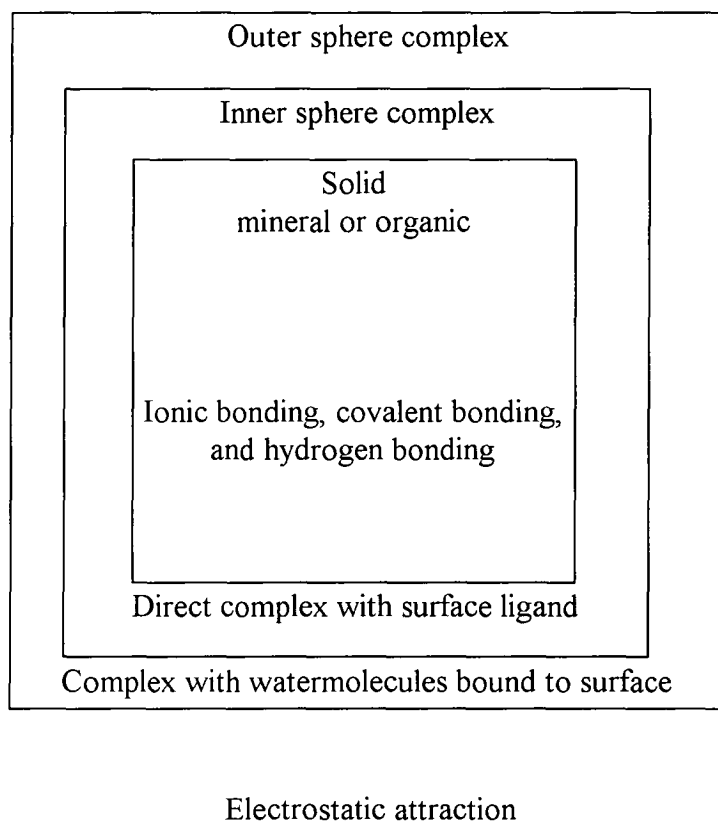


Figure 2.1: Arrangement of layers surrounding a soil solid in contact with the soil solution. At the bottom of the boxes the forces acting on atoms and ions are given. The size of the boxes does not represent the actual relative sizes of the layers.

2.2.2 Interaction of Cu and Zn with soil solids and soluble ligands

Only a small proportion of Cu and Zn are held in the CEC and they are more likely to be specifically adsorbed to the soil solids (McLaren & Crawford, 1973a; Shuman, 1979, 1985). This type of bonding depends strongly on the nature of the metal ion and the surface group and is more specific and less reversible than the attraction to the CEC. Ions are adsorbed in coordination complexes either directly with the surface of the solid or to the ice-like layer of water adsorbed to the surface. These complexes are known as inner sphere and outer sphere complexes respectively (McBride, 1994). The extent of specific adsorption is very pH dependent, because it depends on the presence of free electron pairs on surface sites, which are reduced by protonation.

Cu and Zn form strong complexes with soil colloids and their solution concentration is normally too low to be buffered by the solubility product of solid minerals (McLaren *et al.*, 1981; Barak & Helmke, 1993). Their availability is controlled by the equilibria of adsorption-desorption processes involving inorganic (main ligands for Zn) and organic (main ligands for Cu) solids. The colloidal fraction of the soil is particularly important in these processes due to the high surface area to volume ratio. Cu and Zn concentrations are controlled by the pH of the soil solution. In acid soils their concentration in solution is inversely related to the pH, because adsorption both specific and electrostatic is inversely proportional to the protonation of functional groups at the surface of particles.

The amount of Cu bound by the CEC is small yet well buffered (McBride, 1981). In a desorption experiment with New Zealand soils, ten consecutive desorptions with 0.01 M CaCl₂ desorbed only a fraction of the Cu extracted with EDTA (Hogg *et al.*, 1993).

In most of the soils studied in this experiment, the desorption isotherm reached a plateau after these ten desorptions, indicating that the directly exchangeable pool of Cu had been exhausted. Specific adsorption of Cu occurs on colloidal soil material comprising organic matter, clay and free oxide. Cu adsorbed in this way can be desorbed with dilute acetic acid, but cannot be exchanged with 0.01 M CaCl_2 , though part of it may be exchangeable with Pb or another Cu isotope (McBride, 1981). The specifically adsorbed Cu is assumed to be bound to the soil surface in outer (exchangeable) and inner (non exchangeable) sphere complexes. Organic matter, clay and iron oxide show an increased adsorption of Cu with pH, while adsorption on Mn oxide is pH independent (McLaren & Crawford, 1973b). A major fraction of the soil Cu is comprised of organic complexes which are considered to be the main pool of plant available Cu (Stevenson & Fitch, 1981). Complexing can both increase and decrease the availability of Cu in the soil depending on the solubility of the complexes. Fulvic acids, as part of the dissolved organic matter, are considered important in the mobilisation of Cu as they are more soluble than the larger humic acids (Stevenson & Fitch, 1981). The dissolved chelating agents have a variable influence on the adsorption of Cu onto soil colloids. Humic acid decreased adsorption onto kaolinite (Gupta & Harrison, 1982), while fulvic acids increased adsorption (Dalang *et al.*, 1984). The adsorption of Cu onto artificial silicate surfaces was enhanced by the presence of low molecular weight chelating agents (Elliott & Huang, 1979).

The adsorption of Zn to soil components is generally weaker than that of Cu, but it still determines the concentration of Zn in the soil solution. The effect of pH on the adsorption of Zn is so strong that pH has been termed the 'master variable' controlling the solution concentration (Msaky & Calvet, 1990). Apart from the effect of pH on the

availability of surface ligands for complex formation, it also affects the proportion of Zn present as Zn(OH)^+ . In the pH-range normally encountered in soils, the concentration of Zn(OH)^+ increases tenfold with each unit increase of pH (Barrow, 1993). Zn(OH)^+ has been implicated in the reaction of Zn with soil (Tiller *et al.*, 1972) and the proportion of Zn present as that ion is therefore important. Plotting of Zn adsorption vs. the concentration of Zn(OH)^+ and the effect of pH on this relationship was used to separate adsorption due to disassociated surface ligands and that due to electric charge (Barrow, 1993).

The metal ions in the soil solution are present as free ions and as complexes with soluble ligands. The major ligands participating in reactions with free ions are dissolved organic matter such as fulvic acids. The proportion of Cu as the free ion is lower than that of Zn (Sanders 1983). More than 99% of Cu was bound to soluble ligands in displaced solutions after incubation of soil with water for eight weeks (Sanders, 1982). By contrast up to 80 % of total Zn was present in free ionic form after 88 days incubation (Sanders, 1982). Differences in the proportion of free metal ions depended largely on the original pH of the soils (Sanders, 1982, 1983). A change of the soil pH by the addition of calcium hydroxide also had an effect on the proportion of free Cu and Zn. An increase of one pH unit reduced the proportion of free Cu approximately tenfold from 0.1% to 0.008 % (Sanders, 1982). The proportion of free Zn decreased from 75 to 25 % when the pH increased from 5.40 to 6.15, but a further increase to 6.60 had no effect (Sanders, 1983).

2.2.3 The soil/plant system

The relationship between the plant roots and the soil surrounding them can be envisaged as a system of components in dynamic equilibrium with each other. Fluxes of water and nutrients occur between the different soil components (Figure 2.2). Depending on thermodynamic stability and reaction kinetics, these components act as sources or sinks. Depletion or accumulation of nutrients can result in the change of status of these components. Movement of water follows water potential gradients towards the root surface from where it is taken up into the root and transported via the xylem to the transpiring leaves. A proportion of the nutrients is supplied by the mass flow while the remainder is supplied by diffusion. When demand by the plant is higher than the supply by the soil, a concentration gradient develops inducing nutrient flux to the roots by diffusion. However, some nutrients have a mobility in the soil that exceeds their uptake by plants. As a consequence, they can accumulate near the root. An example is the precipitation of gypsum crystals on roots (Barber, 1995). When nutrients accumulate near the root surface, the direction of diffusion will oppose that of mass flow. Radial transport of the ions from the root surface to the xylem follows two alternate pathways. In one pathway ions move through the apparent free space of the root apoplast; in the other ions move symplastically through the cytosol and plasmodesmata of the root parenchyma tissue. The apoplastic pathway is interrupted at the endodermis by the impermeable Casparian Strip and it is generally accepted that ions have to cross the cell membrane of root cells at least once. Direct transport through the apoplast to the xylem is possible where lateral roots disrupt the endodermis, as in some species, via passage cells (Clarkson, 1991).

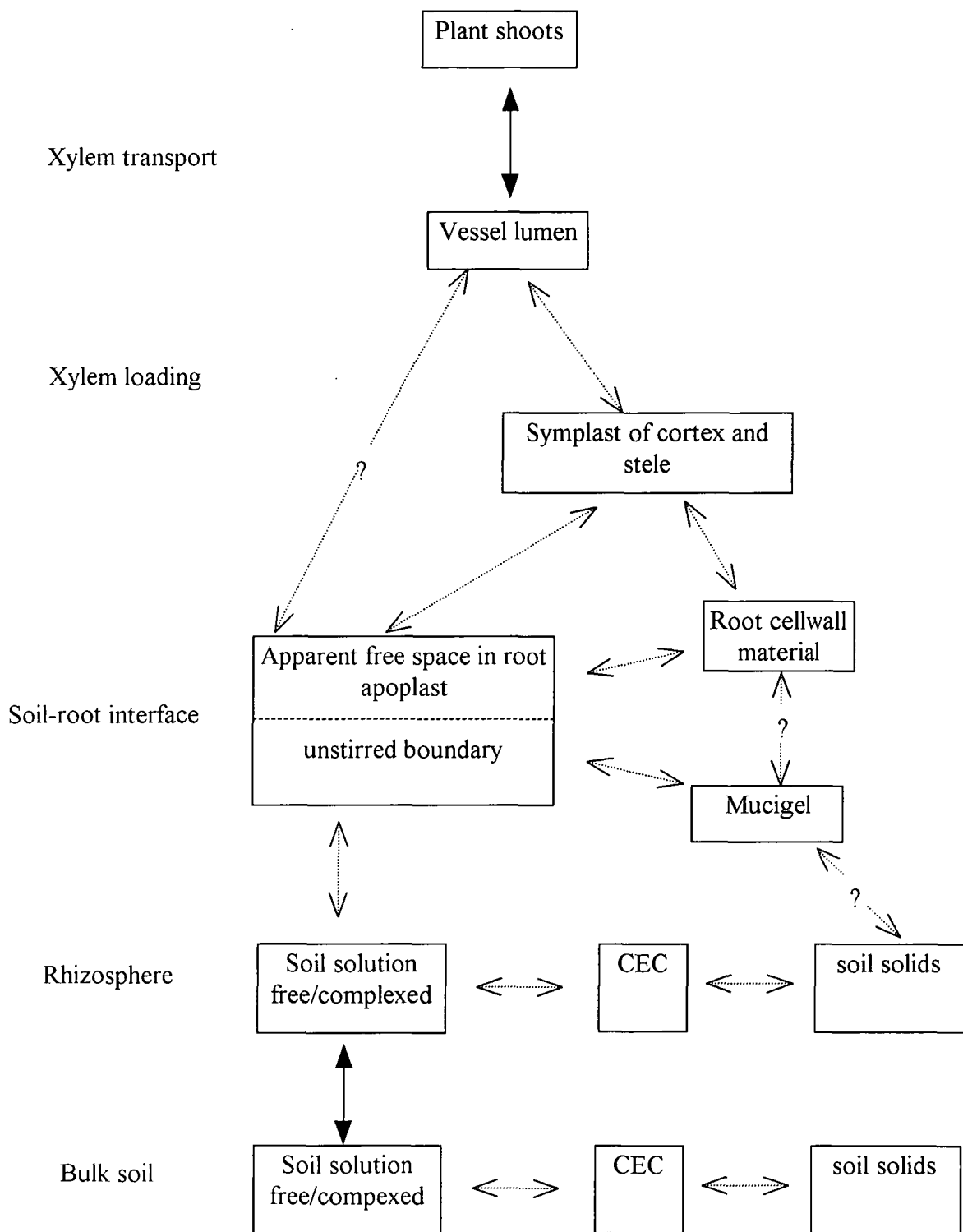


Figure 2.2: The soil / plant system as a system of interacting components. The components are in a dynamic equilibrium with each other. Closed arrows indicate interaction by convection and dashed arrows interaction by diffusion.

2.2.4 Cu and Zn uptake by plant roots

Little research has been conducted into the uptake of micronutrients by plant roots (Graham, 1981; Kochian, 1993). Research has likely been hampered by the low concentrations present in natural systems. Both Cu and Zn show biphasic uptake kinetics starting with a rapid phase which has been attributed to adsorption to cell wall materials through cation exchange (Kochian, 1993; Veltrup, 1976). It is not known whether this adsorption is a necessary first step in the uptake of Cu and Zn into plant roots. Similarly, it is not clear whether Cu and Zn uptake is metabolically dependent: some researchers report inhibition by anaerobiosis, low temperature and metabolic inhibitors, while others found no effect (Graham, 1981; Kochian, 1993). An area where there have been some interesting developments in recent years is resolving which species of metal ion is responsible for controlling the uptake into plant roots. Since DeKock & Mitchell (1957) found that chelators reduced the uptake of divalent metals, it has been assumed that the free metal ion is the determining species. The 'free-ion activity model' has been generally accepted although it has since been shown that an increase in plant concentration can occur with higher total metal ion concentration in solution when the free ion activity was kept constant with chelators (Laurie *et al.*, 1991 a, b). Knowledge of the ionic species is important, as studies determining the critical ion concentration for ion uptake (for example Norvell & Welch, 1993) used chelator-buffered nutrient solutions to achieve low free ion activities. Using the GEOCHEM-PC database, Parker & Pedler (1997) simulated the effect of changing parameters on the importance of the free-ion activity model on metal uptake. They found that the equilibrium of the ligand/free ion/cell surface system was controlled by the soluble ligand only under two conditions. Firstly a large solution/root ratio resulting in a low effective concentration of the surface ligand, and secondly a high

chelating strength of the soluble ligand. Both these assumptions are generally valid in the nutrient solution system, but do not apply to the conditions in the rhizosphere. The results of this simulation demonstrate the difficulty of extrapolating from the simple solution system to the more complex soil system when dealing with a nutrient with a complex chemistry like Cu.

2.3 Effect of N and P nutrition on Cu and Zn uptake by plants

2.3.1 Interaction of Cu with N and P

High levels of N have been shown to both increase or decrease concentrations of Cu in plant shoots. High N nutrition promoted the uptake of Cu in *Persea gratissima* leaves (Lahav & Kalmar, 1990). High levels of N fertilisation led to Cu deficiency in *T. aestivum* after Cu had been applied previously at lower than recommended levels (Brennan, 1993). In another study using *T. aestivum*, Singh & Swarup (1982) reported an increase of Cu concentration in the harvested straw with increasing N without applied Cu, but an increase of Cu both in the straw and the grain when Cu was applied as well. Cu concentration of leaves of *Oryza sativa* was reduced by high N in solution culture (Dias & Oliveira, 1996). Application of N (100 mg/kg) in combination with high P resulted in a reduction of foliar Cu in *Pinus elliotii* var. *elliottii* (van Lear & Smith, 1972). Application of 50 mg/kg N to an acid podzol without added Cu increased Cu concentration in several forest trees, but higher levels of application resulted in a reduction (van den Burg, 1983). When Cu was applied in that study, Cu concentrations of *Quercus robur* and *Fagus sylvatica* were higher in plants receiving high levels of N than in plants receiving no N.

Decreases in Cu concentration are usually explained as a dilution effect. Increases in concentration with increasing N may be due to suboptimal rates of Cu transport to shoots at low levels of N. Most of the Cu in the xylem sap occurs as a complex with nitrogenous compounds such as asparagine and histidine, which were calculated to account for more than 95% of Cu present in the xylem sap of *Glycine max* (White *et al.*, 1981). Only in complexed form can high solution concentrations of Cu in the xylem sap be maintained, as otherwise it would be adsorbed to the xylem cell walls. Some nitrogenous compounds may also act as specific transport molecules. For example, nicotianamine (NA) has been postulated as responsible for metal micronutrient transport in xylem of *L. esculentum* (Pich & Scholz, 1996) and phloem of *Ricinus communis* (Schmidke & Stephan, 1995). The interaction of N and Cu application on transport and relocation of Cu are discussed more fully in Chapter 6

High levels of P fertilisation can interfere with the uptake and utilisation of Cu by plants and can lead to reduced biomass, the expression of deficiency symptoms and lowered micronutrient concentrations in plant tissues. Application of 25 mg/kg P to *T. aestivum* increased Cu concentration, but application of more than 50 mg/kg P reduced the Cu concentration (Shukla & Singh, 1979). Smilde (1973) reported also that P application interfered with the uptake of Cu in several tree species only when excessive amounts of P were applied. All rates of P application reduced the Cu concentration of *Trifolium subterraneum* (Reuter *et al.*, 1981). A reduction of Cu concentration in the xylem sap of *Pinus maritima* due to P fertilisation which was subject to seasonal variation has been reported (Saur *et al.*, 1995). Significant differences of Cu concentration in the xylem sap due to P application were not always accompanied by differences in foliar Cu concentration. Hence, Saur, *et al.* (1995)

considered the xylem sap concentration a better indicator of P-induced Cu deficiency in *P. maritima* than foliar analysis.

2.3.2 P-induced Zn deficiency

The interaction between P fertilisation and Zn deficiency is usually termed P-induced Zn deficiency (Ohlsen, 1972). This interaction was first demonstrated more than sixty years ago (Barnette *et al.*, 1936 in Ohlsen, 1972) and has been the subject of more recent study. Wallace *et al.* (1978) reported that more than 100 papers had been published on this interaction between 1970 and 1978. Most research has been concentrated on a number of annual crop plants. There has been one intensive study of P-induced micronutrient disorders in a *Populus* hybrid (Timmer & Teng, 1990). Notwithstanding this large volume of information, it is often conflicting and there is no simple mechanistic basis for this complex interaction.

Interaction between Zn and P have been shown to occur at a number of levels in the soil-plant system (Loneragan & Webb, 1993; Robson & Pitman, 1983):

- Reduced availability of Zn in the soil due to precipitation and adsorption by high P.
- Reduced Zn uptake by the plant due to reduced root length and/or mycorrhizal infection at high P (Davies & Lindermann, 1991).
- Accumulation of the Zn in the root and reduced translocation to the shoot (Warnock, 1970).
- Lower tissue Zn concentration due to dilution by increased biomass production when P is supplied.

- An increased demand for the micronutrient due to a decrease in physiological availability of Zn when internal P levels are high (Cakmak & Marschner, 1987; Leece, 1976)
- Enhanced uptake of P in Zn deficient plants (Marschner & Cakmak, 1986). This may lead to the expression of P toxicity symptoms, which may be mistaken for Zn deficiency symptoms (Loneragan *et al.*, 1979).

2.4 Mechanisms of fertiliser induced Cu and Zn deficiency

High levels of one nutrient are often associated with a reduction in the concentration of another. This may occur because soil conditions, such as pH and redox potential, resulting in high availability of one nutrient may inhibit the uptake of another. Increased growth rate of the plant due to the application of one nutrient can lead to the dilution of a second, again possibly leading to deficiency of that nutrient (Jarrell & Beverly, 1981). There are also specific interactions which lead to increased adsorption and changes in the rate of diffusion in soil, effects on root system architecture and mycorrhizal infection, interference in uptake, and transport by competition, and the formation of insoluble complexes within the plant (Robson & Pitman, 1983).

The role played by several soil and plant factors in the interaction between N and P nutrition and Cu and Zn uptake will now be discussed in more detail. With the exception of mycorrhizae, the research in this thesis will investigate how these factors influence the Cu and Zn nutrition of highly fertilised eucalypt seedlings. These factors are:

- 1) The dilution effect

- 2) Effect of P application on root architecture and mycorrhizal infection
- 3) Effect of P application on adsorption and soil diffusion

The effect of N application on Cu transport in the plant will be discussed in the Introduction of Chapter 6.

2.4.1. Dilution effect

The concentration of a nutrient in plant tissues depends ultimately on the relationship between the rate of uptake of the nutrient into that tissue and its growth rate. This interdependence between concentration and growth rate leads to an effect known as the ‘Steenbjerg’ effect, the reason for the sometimes C-shaped nutrient response curve of yield (Bates, 1971). This is caused by the increase in growth rate when marginal amounts of a given nutrient are applied to a plant deficient in that nutrient which is then diluted by the increase in biomass. A pronounced dilution of micronutrient concentrations may also occur when a limiting macronutrient is applied. This dilution effect has been reviewed by Jarrell & Beverly (1981) and it has implications for the analysis and interpretation of nutrient experiments. For example, measurement of dry mass and calculation of total accumulation are necessary to show whether a dilution (or concentration) effect has occurred. For this reason Imo & Timmer (1997) used vector analysis with relative biomass and nutrient concentration as the coordinates as the most appropriate way to interpret nutrient responses.

A specific effect of changes in the growth rate due to nutrient application are changes in the shoot : root ratio of plants. Increases in shoot : root ratio with greater availability of N have been reported previously (eg. Ericsson, 1981, 1995; Ingestad & Kaehr, 1985; Misra *et al.*, 1998). Thornley (1972) developed a model that explained

the response of shoot : root ratio to nutrient addition. The model relies on the resistance to transport of N from the root to the shoot and of photoassimilate from the shoot to the root to explain changes in shoot : root ratio. The tissue further away from the source of a nutrient (ie. N for the shoots and carbohydrates for the roots) will be affected more strongly when the nutrient becomes limiting. The response of the shoot : root ratio to major nutrients conforms well with this model (Wilson, 1988). The reduction in the relative size of the root system may lead to a reduction in soil exploitation volume and decreased uptake of the soil immobile nutrients like Cu and Zn. This may lead to deficiency in soil with a marginal supply of these elements.

Nutrients	Approximate percentage nutrient uptake through:		
	Interceptive root growth	Mass flow	Diffusion
N	2	98	--
P	3	6	91
K	2	20	78
Ca	28	72	--
Mg	13	87	--
S	5	95	--
B	3	65	32
Cu	70	20	10
Fe	50	10	40
Mn	15	5	80
Mo	5	95	--
Zn	30	30	40

Table 2.1: Percentages of nutrient uptake through roots accounted for by interceptive root growth, mass flow and diffusion. Compiled from data in Barber & Ohlsen 1968, Dennis 1971. (Table from Bergmann 1992 p.26)

2.4.2 Effect of root system size and mycorrhizal infection on the plant uptake of Cu and Zn

The extent to which a root system exploits soil volume has marked influence on the uptake of relatively soil-immobile elements like Cu. Although for most nutrients mass flow or diffusion are the main source of nutrient supply roots, in the case of Cu root interception is necessary (Table 2.1, Bergmann, 1992). It is arguable, that for

nutrients like Cu which have very low concentrations in the soil solution and form stable complexes with soil particles, the importance of direct ligand exchange between root and soil particles may be more important than that of diffusion driven by a small concentration gradient. P deficiency had a stimulating effect on the growth rate and length of root hairs of *Arabidopsis thaliana* (Bates and Lynch, 1996) and on tap roots and lateral roots of *Macrotylamo uniflorum* (Anuradha & Narayanan, 1991). Uptake of micronutrients was not reported in either of these studies.

Infection with mycorrhizae has been shown to increase the acquisition of minerals by infected plants, a process thought to occur due to the greater soil volume exploited and the release of fungal substances into the rhizosphere. Mycorrhizal weathering of primary soil minerals has been demonstrated and it has been suggested that 'rock-eating' hyphae can contribute to plant nutrient uptake more directly than uptake via the bulk soil solution (van Breemen *et al.*, 2000). Increases in the plant uptake of P and micronutrients by mycorrhizae have been shown by a number of researchers (Gnekow & Marschner, 1989; Kothari *et al.*, 1991; Pacovski, 1986). The contribution of VAM fungi to Zn uptake in a split pot experiment ranged from 15 to 48 % depending on the application of P and Zn. In the same experiment Cu concentration in the shoots was not affected by Cu application to the hyphal compartment of the pot, although the root concentration increased by more than 100 % (Kothari *et al.*, 1991). This difference may be caused by an increase in shoot, and a decrease in root, dry mass in response to the treatment. Mn concentration in the roots and shoots was reduced in response to application of Cu, Zn and Fe to the hyphal compartment, indicating competition of one of the applied nutrients with the hyphal uptake of Mn (Kothari *et al.*, 1991).

Hetrick *et al.* (1996) showed that the yield response of *T. aestivum* to mycorrhizal inoculation was reduced with increasing P supply and reached zero between 40 and 50 mg/kg P added. Mycorrhizal infection of *Pinus resinosa* by *Hebeloma arenosa* was also reduced with increasing P nutrition and at the highest P treatment (136 mg/kg added) no infection occurred (MacFall *et al.*, 1991). Increasing levels of P nutrition were associated also with reduced per cent root length with VAM colonisation in *Capsicum annuum* and lower Cu and Zn concentrations in the tissues (Davies & Lindermann, 1991). Mycorrhizal infection of maize roots was not affected by P fertilisation, but the length of hyphae was reduced significantly by high P application (Kothari *et al.*, 1991). However, no effect of P nutrition on the concentration of Cu and Zn in the maize plants was reported.

2.4.3 Effect of P application on Cu and Zn adsorption to soil solids and their diffusion in the soil

The precipitation of metal cations as insoluble phosphates is a well known phenomenon and is the major mechanism by which Al toxicity can be ameliorated with P fertilisation (Robson & Pitman, 1983). Direct precipitation of Cu with phosphate in the soil as a mechanism for reduced Cu uptake is unlikely, as the concentration of Cu in the soil solution is controlled by adsorption on organic complexes (McBride, 1981). High P fertilisation may reduce the availability of Cu in the soil by increasing the adsorption of Cu onto colloidal surfaces (McBride, 1985). Like Cu, Zn undergoes a number of adsorption/desorption reactions in soil. The relative importance of these reactions in the control of Zn availability in soil is higher (Bruemmer *et al.*, 1983) than direct precipitation/dissolution reactions. Phosphate anions have been shown to increase the adsorption of Zn on goethite surfaces slightly

(Bolland *et al.*, 1977). Melton *et al.* (1973) showed that the diffusion coefficient of Zn was less reduced by addition of P than by an increase in soil pH. As P fertilisation often acidifies the soil (Al-Showk, *et al.*, 1987), it results in higher Zn availability.

2.5 Diagnosis of Cu and Zn deficiencies in plant nutrition

The knowledge of the importance of a number of nutrients for plant health led to the development of diagnostic methods used to identify nutrient disorders in plants. The results of these methods can be verified by a response of plant growth to the supply of a nutrient either in the field or in the glasshouse. The early history of the diagnosis of nutrient disorders in plants and early diagnostic methods has been extensively reviewed by Goodall and Gregory (1947). They divided the methods into three groups:

- 1) Soil analysis
- 2) Diagnosis of deficiency symptoms
- 3) Plant analysis

This division is still relevant (Reuter & Robinson, 1997) and each of the three groups will be briefly reviewed below.

2.5.1 Soil analysis

The advantage of using soil analysis to predict plant uptake of nutrients is that it is possible to identify fertiliser needs prior to planting. Therefore there will be no reduction of growth and it will be easier to ameliorate problems. The main problem with soil tests is to identify the proportion of the nutrient that is available to the plant and how well it is represented by the extractant. The 'plant available' fraction of the

nutrient depends on the soil type and the plant species used. It may therefore be necessary to calibrate critical concentrations for different soil-plant combinations. It was found early on that the total nutrient concentration was only poorly correlated to the concentration in plants grown on that soil (Goodall & Gregory, 1947). Early chemical methods to identify the available nutrient fraction, including weak acids and dilute salt solutions, have been reviewed (Piper, 1942).

Extraction of nutrients by biological means was another way to extract the plant-available nutrient fraction. Seedlings of *Secale cereale* were grown on a limited amount of soil for eighteen days and then analysed. The amount of nutrient extracted by the seedlings after subtraction of that found in plants grown on pure sand, was considered root-soluble (“wurzellöslich”) (Neubauer, 1923). Other researchers have used a variety of microorganisms to estimate the plant-available nutrient status of soils. Biological methods were not found to give superior results to chemical extractions and did not gain widespread use, because of their time consuming nature (Goodall & Gregory, 1947). Once chelating agents started to be used in plant nutrition research, it was found that they gave good approximations of the plant uptake of metal micronutrients such as Cu, Mn and Zn. The advantage of these methods is that they can extract several micronutrients at once. The most common methods today for the extraction of plant-available Cu, Mn and Zn (Rayment & Higginson, 1992) use the chelators DTPA (Lindsay & Norvell, 1978) and EDTA (Clayton & Tiller, 1979) either alone or in combination with dilute salt solutions and acids (Mehlich, 1984) .

2.5.2 Visual deficiency symptoms

The diagnostic use of visual deficiency symptoms does not rely on laboratory equipment and is therefore an ideal field test for nutrient deficiencies. A large number of books have been published containing descriptions and/or colour plates of nutrient deficient crop plants (Bennett, 1993; Bergmann, 1992) and *Eucalyptus spp.* (Dell *et al.*, 1995). A major disadvantage of the use of visual symptoms is that deficiencies of different nutrients may have very similar symptoms and that sometimes deficiencies or toxicities in one nutrient will express their symptoms through their effect on another nutrient. Also by the time visual symptoms appear, it may be too late to effectively ameliorate chronic deficiency or even mild disorders. Marginal deficiencies may also not result in the expression of deficiency symptoms, yet may still lead to a reduction in yield or quality.

2.5.3 Plant analysis

The most direct measure of the plant's nutritional status is the analysis of the plant material itself. A large number of experiments have been conducted linking the nutrient concentration in the plant to the potential growth response to nutrient addition. The results of these experiments have been tabulated listing deficient, adequate and toxic concentration ranges, as well as other significant concentrations (Bergmann, 1992; Chapman, 1966; Reuter & Robinson, 1997). The interpretation of the results of plant analysis is hindered by the large variation in most nutrient concentrations. The concentrations vary seasonally, with the age of the plant and between different plant tissues. Care must therefore be taken to standardise the methods used. For some micronutrients, the total nutrient concentration may not be indicative of the potential response of the plant to nutrient addition, such as in some cases of P-induced Zn

deficiency (Leece, 1976). To overcome this problem some researchers used analysis based on nutrient ratios like the DRIS system developed by Beaufils (1973). It also may be difficult to detect a temporary nutrient deficiency by plant analysis, particularly if it occurs early in the growth of the plant (Bell, 2000). Bell quotes research by Mulyati *et al.* (1997) which showed that early Zn deficiency in transplanted *Brassica napus* seedlings depressed yield potential, but was not detected in later leaf analysis.

Another difficult aspect of plant analysis is to define the concentration indicating that deficiency is present. A graph plotting biomass against nutrient concentration shows an increase of biomass with higher nutrient concentration in the deficient range. Biomass will reach a plateau when the nutrient concentration is adequate and further increases in nutrient concentration will not lead to an increase in biomass (Bates, 1971). The level at which the plant reaches 90% of its maximum biomass has been termed the critical concentration and is generally used as the boundary between deficient and adequate concentrations. Due to a number of factors that influence the value of the critical concentration, including genetic and climatic differences, it is usually better to assume a critical range rather than a particular concentration (Smith and Loneragan, 1997).

Some researchers have investigated the activity of micronutrient-containing enzymes and found that they gave a good indication of the nutrient requirement of the plant (Brown & Hendricks, 1952; Gherardi *et al.*, 1999; Loneragan *et al.*, 1982; Rao & Ownby, 1997). This also allowed the development of a quick field test for Cu deficiency in *T. subterraneum* using the activity of ascorbic acid oxidase (Delhaize *et al.*, 1982).

2.6 Methods used in research into Cu and Zn nutrition

2.6.1 Methods of nutrient supply

There are two ways in which nutrient experiments with plants can be conducted. Plants can be either grown in soil or in nutrient solutions, either directly or in solutions applied to an inert medium like sand or gravel. The growing of plants in well-defined nutrient media has been an invaluable tool in plant nutrition research. Solution experiments have been used to prove the essentiality of a nutrient, as they minimise contamination in nutrient exclusion experiments. The reduction in the heterogeneity of the root/nutrient medium interface allowed the presentation of a defined nutrient concentration to the root, facilitating studies in the kinetics of nutrient uptake. Methods used by early researchers have been reviewed in detail by Hewitt (1966) and the latest advances in solution culture techniques by Parker & Norvell (1999). The review in this chapter will therefore concentrate only on several potential problems with solution culture experiments.

The concentration of the nutrient in the free space- of the root ultimately will influence its uptake into the plant. This surface is supplied from the bulk solution by diffusion and generally a depletion zone will form around the root leading to a change of concentration of the root bathing solution. To overcome this problem, the need for stirring of the solution (Ohlsen 1950, 1953) or a high solution flow rate (Edwards & Asher, 1974) has been emphasised. The presence of an unstirred boundary layer even in turbulent flow has been demonstrated by Polle and Jenny (1971), who assumed that the uptake of Rb into barley roots was a function of an unstirred layer around the root. The extent of the unstirred layer is inversely related to the turbulence of this system.

The problem of changing concentrations in the nutrient solution during plant uptake also has implications for the whole system. As the plant takes up nutrients, the pool of available nutrients in the solution decreases slowly. To avoid problems with a changing concentration, high solution concentrations or large volumes were used to make the available pool of nutrients very much larger than the amount taken up by the plants. Large vessels cause logistic problems and result in large and complicated set ups (Hewitt, 1966) that may restrict the size of the experiments conducted. The use of high concentrations in traditional solution culture to maintain the concentration constant has been criticised, as this may lead to artefacts of the method where roots are presented with solutions of much higher concentration than the soil solution (Ingestad & Ågren, 1992). It has been shown that high growth rate can be achieved with low external concentrations and frequent nutrient additions (Ingestad, 1982). This system achieves steady state nutrition where the relative nutrient addition rate is closely linked to the relative growth rate and concentrations in the soil/plant system stay essentially the same. A comparison between the two methods of nutrient supply (solution concentration vs. addition rate) has been recently undertaken which showed that the method of nutrient addition impacted on the conclusiveness of the experimental results (Hellgren & Ingestad, 1996). The criticism of the high concentrations used in nutrient solutions in comparison to the soil solution cannot be left without comment, as the data available for the nutrient concentration in soil is based on average values of soil extracts. This situation may differ from what the root experiences in the field, as it grows through a number of micro-environments which may contain much higher concentrations than the bulk solution. The effect of root exudates and the microbiota in the rhizosphere combined with environmental influences, especially wetting and

drying cycles, may lead to uptake from more concentrated solutions than those found in soil extracts.

Unlike solution cultures, soils have a high capacity to store nutrients and deliver the nutrients with low intensity. They are therefore easier to manage in nutrient studies. Nutrients can be supplied either as a batch at the beginning of the experiment or as a continuous supply, usually by addition of nutrient solution. When working with micronutrients, it is important to apply the major nutrients as analytical reagents which are free of contamination with micronutrients. Nutrients that are added as a batch have to be homogenised well with the soil and it may be necessary to incubate the pots for some time to allow the soil to react with the nutrient and to avoid possible damage to sensitive young seedlings. Although easy to set up, experiments using soil have two major disadvantages: the removal of soil adhering to roots during harvest and the poor definition of the nutrient concentration supplied to the plant root.

2.6.2 Analytical methods

The analysis of soil and plant materials for their micronutrient content is difficult due to their low concentration which may be below the detection limit of analytical equipment. This is particularly true for studies of nutrient deficiencies. In the absence of very sensitive chemical methods for some elements (eg. Mo), biological methods were employed in the past. It was found that some nutrients were essential for the growth of micro-organisms such as *Aspergillus niger* (Nicholas, 1952). Incubation of *A. niger* on growth medium with a standard amount of the material being tested was used to measure concentrations by comparison with a standard curve. Contamination in nutrient solutions could also be detected with this method. At the same time a

number of colorimetric methods were developed. These methods measure the colour of organic complexes with metals; for example carbamate with Cu and dithizone with Zn. Although these methods were sensitive, they were time consuming and subject to interference with other metals (Allen, 1989). With the improvement of spectroscopic methods, the biological and colorimetric methods were superseded by spectroscopy. Early instruments had low sensitivity and for the analysis of low concentrations, extraction of metal complexes into small amounts of organic solvent were often necessary (Allen, 1989). Modern instruments have much better sensitivities and Atomic Absorption Spectrometry (AAS) either by flame or by graphite furnace has become the most often used analytical method for Cu, Mn and Zn. Atomic Absorption Spectroscopy has been reviewed in great detail by Kirkbrecht & Sargent (1974). Even greater sensitivity can be achieved by the use of Inductively Coupled Plasma-Mass Spectroscopy (Soltanpour, 1996). A great advantage of this method is that a large number of elements can be analysed at the same time, but the latter instrument is extremely expensive and not always readily available or affordable for diagnostic work. Most of the colorimetric and spectroscopic methods need the sample in a liquid form without solid particles. Preparation of samples consists of either digestion of solid samples with a mixture of strong mineral acids and oxidising materials (Lowther, 1980; Xing & Venneman, 1998) or dry ashing in muffle furnace (Allen, 1989). Liquid samples such as natural waters or soil solutions, can usually be directly analysed, although removal of colloidal materials and/or the oxidation of dissolved organic matter may be necessary.

2.7 Conclusions

The research reviewed in this chapter shows that N and P application has resulted in induced Cu and Zn deficiencies in a number of plants. This phenomenon is well studied and interactions were shown at different levels in the soil/plant system. They occur during:

- Soil supply of the root
- Uptake into the root
- Root to shoot transport
- Dilution during growth
- Physiological availability

There is little knowledge about fertiliser-induced Cu and Zn deficiencies in eucalypts. The research in this thesis will investigate the effect of N and P application on growth and Cu and Zn nutrition of *E. nitens* seedlings in the greenhouse under controlled conditions and attempt to increase the knowledge in that aspect of eucalypt nutrition. In particular the effect of N and P application on root growth and shoot:root ratio, Cu and Zn concentration in roots and shoots will be studied. The influence of mycorrhizae on the interaction will not be studied, because the research in this thesis will concentrate on chemical analysis and not mycological research.

Considering the advantages and disadvantages of the two methods of nutrient supply reviewed above, it was decided to use soil in the experiments of this thesis. There are problems transferring results from solution experiments for nutrients like Cu and Zn, which are strongly affected by adsorption phenomena in the quite different soil environment. It is also difficult to determine the concentration range that should be adopted, when the research is attempting to determine the interaction between N and P

application and Cu and Zn nutrition. Soil offered the best approach to this problem. Since the soil used in the experiment had been shown to result in Cu-deficient plants in the field, it was reasonable to assume that it would be possible to grow Cu-deficient plants in this soil in the greenhouse. It was then only necessary to add Cu and Zn to overcome the deficiency or at least observe their uptake in relation to controlled factors.

Chapter 3: Cu deficiency and stem deformities in fertilised plantation eucalypts - a field study

3.1 Introduction

Stem deformities have been observed in fast growing, fertilised plantation trees. Plantations established on ex-agricultural land are particularly prone to problems with stem form. The stem deformities resemble those associated with symptoms of Cu deficiency and were first described for *P. radiata* (Ruiter, 1969), but have also appeared in eucalypt plantations (Turnbull *et al.*, 1994).

3.1.1 *P. radiata*

Stem deformities in fertilised plantation trees were first reported in Australia for *P. radiata* (Ruiter, 1969) and Cu deficiency was suspected as the cause. Subsequently, studies were undertaken to test this hypothesis and to investigate the anatomical and physiological changes that led to stem deformity (Downes & Turvey, 1992 a). Two causes have been described. One indicated Cu deficiency and was associated with low Cu levels in the needles and reduced lignification (Downes & Turvey, 1986). The other was linked to the application of high levels of N and P on ex-pasture land (Carlyle *et al.*, 1989) and was termed 'Toorour syndrome' after the region where it was first described. It was not accompanied by a reduction either of foliar Cu concentration or lignification (Downes & Turvey, 1990a) and could not be ameliorated by application of Cu fertiliser (Hopmans, 1990). A high degree of genetic

variability in susceptibility to stem deformity has been reported for *P. radiata* (Pederick *et al.*, 1984; Bail & Pederick, 1989).

Although a reduction of lignification was identified as one of the causes of stem deformity associated with Cu deficiency in *P. radiata* (Downes & Turvey, 1986, 1990b), the degree of lignification was not related to the severity of stem deformity. It is not surprising that reduced lignification should lead to a loss of stem form in trees. Lignification may not only be necessary for the structural strength of the upright stem, but is implicated in the gravitropic response of trees. According to the 'lignin swelling model' of the origin of growth stresses (Kubler, 1987), expansion of the macromolecule during the final polymerisation step leads to longitudinal contraction (angiosperms) or extension (gymnosperms) depending on the angle of the cellulose microfibrils in the cell wall. The reaction anatomy of leaning stems results in changes to growth stresses in the stems, which return their orientation to the vertical (Scurfield, 1973). The control of stem form by reaction wood is discussed in detail in Chapter 5.

The cause of 'Toorour syndrome' is not clear and may result from an interaction of a number of factors. Turvey *et al.* (1992) investigated the effect of weather, the ratio between soluble and total N in buds and the Cu concentration in buds on the expression of stem deformity on an ex-pasture site. They found that warm weather and a high level of mineralisable N led to a late season flush and to stem deformities that were probably exacerbated by high winds. Soluble N was higher and Cu lower in fertilised plots, but there was no consistent difference between straight and deformed trees. They concluded that none of the variables investigated could be clearly identified

as a causal factor. It is apparent though, that a reduction in stem strength with high nitrate availability (Downes & Turvey, 1992 b) and fast growth in warm weather make the stem susceptible to bending.

3.1.2 Eucalypts

Similar deformities that relate to application of N and P fertiliser have been described in *E. nitens* (Turnbull *et al.*, 1994) and are now occurring in a number of ongoing fertiliser trials (unpublished data). Stem deformity increased with higher rates of N and P application. The percentage of prunable trees was reduced from more than half to about 15 % at the highest application rates (Turnbull *et al.*, 1994). Turnbull *et al.* (1994) reported a significant linear relationship between the degree of deformity and foliar Cu concentrations below a critical level of 1.4 mg/kg. No deformities occurred in trees with foliar Cu concentrations above 1.4 mg/kg. There was no significant regression relationship between the concentration of other nutrients and the degree of deformity. Similar symptoms of pendulous growth and sinusoidal stem growth have been encountered in a trial with *Eucalyptus globulus* on a sandy soil (Paul Adams, pers. Comm). The trees received a high rate of N fertilisation and the symptoms appeared in the second growing season. The trees responded well to an ameliorating treatment with Cu fertiliser. The symptoms observed are similar to those previously reported for Cu deficiency in *E. nitens*, *E. globulus* (Dell *et al.*, 1995) and *Eucalyptus maculata* (Dell & Bywaters, 1989).

3.1.3 Objectives of the research in this chapter

The previous results suggest that Cu deficiency, induced by high rates of N and/or P application, causes stem deformities. To further investigate the causes of fertiliser-induced stem deformity in *E. nitens*, an analysis of data collected previously by Turnbull and others and a field survey at Gould's Block, their experimental site, were undertaken. The aim of this investigation was to explore the possibility that other micronutrients may interact with Cu in the expression of symptoms. Interaction with other micronutrient deficiencies may be the reason no direct evidence for Cu deficiency was found in trees displaying 'Toorour syndrome'. In particular the possibility that Zn deficiency is involved will be explored, because the inhibitory effect of P application on Zn concentration is well known and Zn deficiency causes dieback of leaders. Also the stem deformities described by Ruiter (1969) occurred on a site with a known Zn deficiency. Another aim of this chapter is to identify possible causes, why trees showing deformities may have reduced Cu concentrations.

The data used included some published by Turnbull *et al.* (1994) which are analysed in a different way to that presented in their paper. The data not previously published shows the effect of N and P application on foliar nutrient concentrations and the results of an amelioration trial with micronutrient application. Three complete trees of differing degrees of deformity were harvested during the field survey to initially investigate the distribution of Cu, Mn and Zn in different parts of the tree. The field survey also provides a more detailed description of the soil including excavation of several root systems of trees with differing degrees of stem deformity. The purpose

was to visually assess the root development of affected trees as a possible cause for reduced Cu uptake.

The research in this chapter tested the following hypotheses:

- Micronutrients in addition to Cu are involved in causing stem deformities induced by high N and P application.
- N and P application reduced the foliar concentration of Cu and/or other micronutrients.
- Application of Cu and/or other micronutrients ameliorated stem deformities.
- Root systems of deformed trees and the soil they grow in, differ from those of straight trees.

3.2 Methods and materials

3.2.1 Site description

The site was a fertiliser trial in a plantation of *E. nitens* north of Dover (43°18'S, 147°01'E) in Southern Tasmania (Turnbull *et al.*, 1994). The 4 ha trial was of a 5²-factorial design in two randomised complete blocks. The treatments were 0, 60, 120, 240 and 480 kg/ha N as urea, and 0, 30, 60, 120 and 240 kg/ha P as triple superphosphate (referred to as levels 1-5 for each element). A more detailed description of the site and the experiment can be found in Turnbull *et al.* (1994). The stem deformity was assessed using a deformity index (DI) (Turnbull *et al.*, 1994). A DI of 1 indicated a straight tree and a DI of 2-6 indicated trees with increasing degree of deformity as described for *P. radiata* by Pederick *et al.* (1984).

3.2.2. Harvest dates and plots sampled previously

3.2.2.1 March 1991 – six months after planting

Foliage samples were taken from 6 trees in each of two plots with the following treatment combinations: N1P1, N2P2, N3P3, N4P4 and N5P5.

3.2.2.2 August/September 1992 – two years after planting

Foliage samples were taken from straight trees in plots with only straight trees and from deformed trees in plots with only deformed trees. In plots with both straight and deformed trees, samples were taken from straight and deformed trees. 7-10 trees from each category were sampled in each plot.

3.2.2.3 Amelioration trial 1992/1993

50 trees were chosen from 5 plots according to their DI. The trees were randomly assigned to 5 treatments:

Treatment 1 – Unfertilised control

Treatment 2 – Complete micronutrient mix applied

Treatment 3 – B applied

Treatment 4 – Cu applied

Treatment 5 – Cu + B applied

The treatments were applied in October 1992.

DI was measured and foliage samples were taken in September 1992, February 1993 and May 1993

The foliage samples were analysed as described in Turnbull *et al.* (1994).

3.2.3 Field survey 1996

3.2.3.1 Soil sampling

Two sets of soil samples were taken. One set was taken to estimate the between and within plot variability of selected soil properties. The other was taken to show differences in soil from the root zone of straight and deformed trees. The leaf litter was removed before sampling. Soil from the top 10 cm was sampled at each site with a stainless steel corer. The soil was sieved through a 2 mm stainless steel sieve and air dried at 40°C.

For the first set, soil was sampled from between rows of duplicate plots with the following treatment combinations: N1P1, N2P2, N3P3, N3P4, N4P3, N4P4 and N5P5. Soil was sampled in a grid at eight points in each plot (Figure 3.1 a). One core from each point was taken. The cores from the eight sampling points were bulked to represent the average soil of the plot. At each sampling point a further ten cores were taken on a 30 cm circle around the central core (Figure 3.1 b). These cores were bulked at each point to represent the soil at each point of the grid. Loss on ignition, pH and EDTA extractable Cu were measured on these soil samples.

For the second set, soil was sampled from the root zone of the trees from which tissue samples were taken and from additional straight and severely deformed trees (see Figure 3.1 a and c). Twenty-five cores were taken within a 1.5 m radius of the tree. The cores taken from the root zone of each tree were bulked. These soil samples were analysed for loss on ignition, pH and micronutrient concentrations after acid digestion.

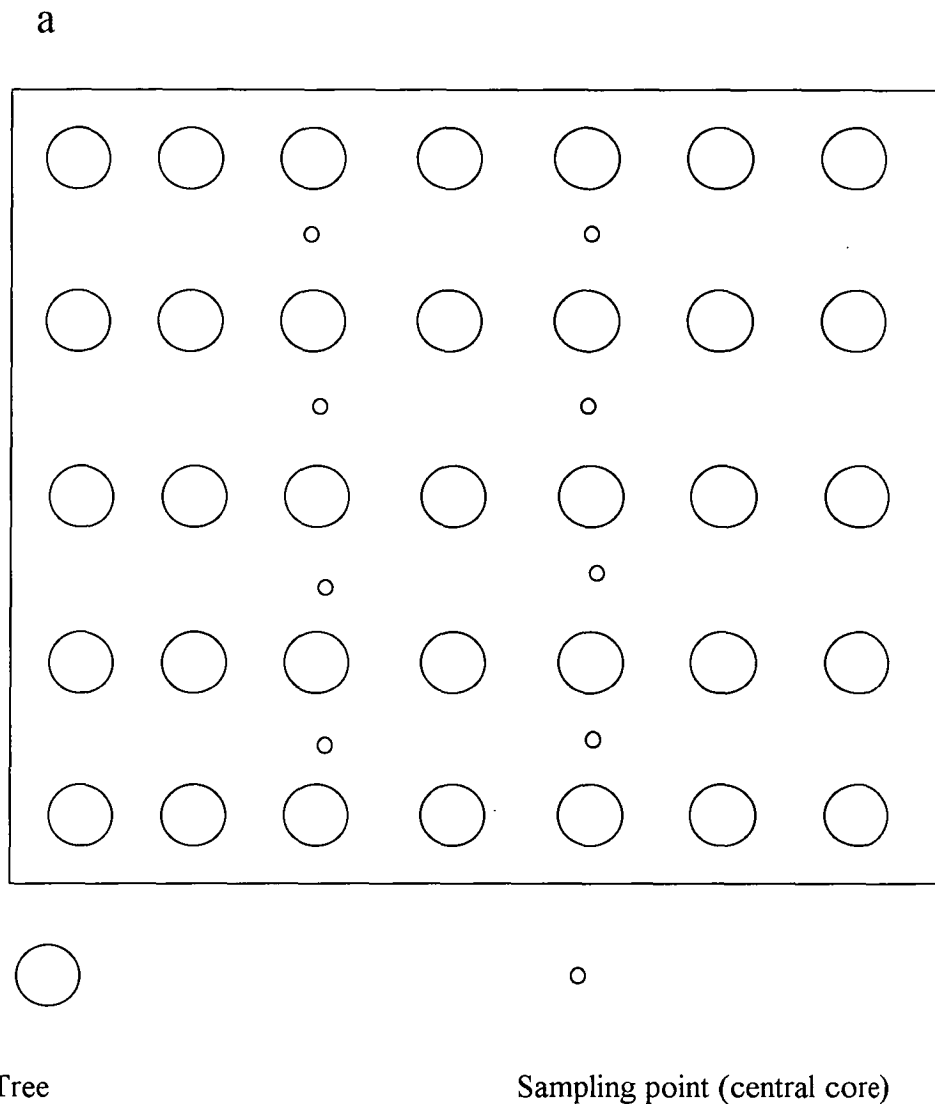
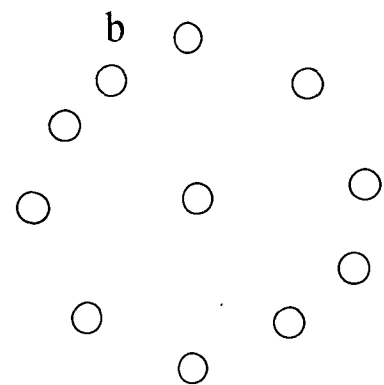


Figure 3.1: Soil sampling strategy for field survey 1996. a) Arrangement of sampling points within the plot. b) Sampling of soil cores around central core. The central core is used for the bulk sample of the whole plot, the others represent the soil at that sampling point



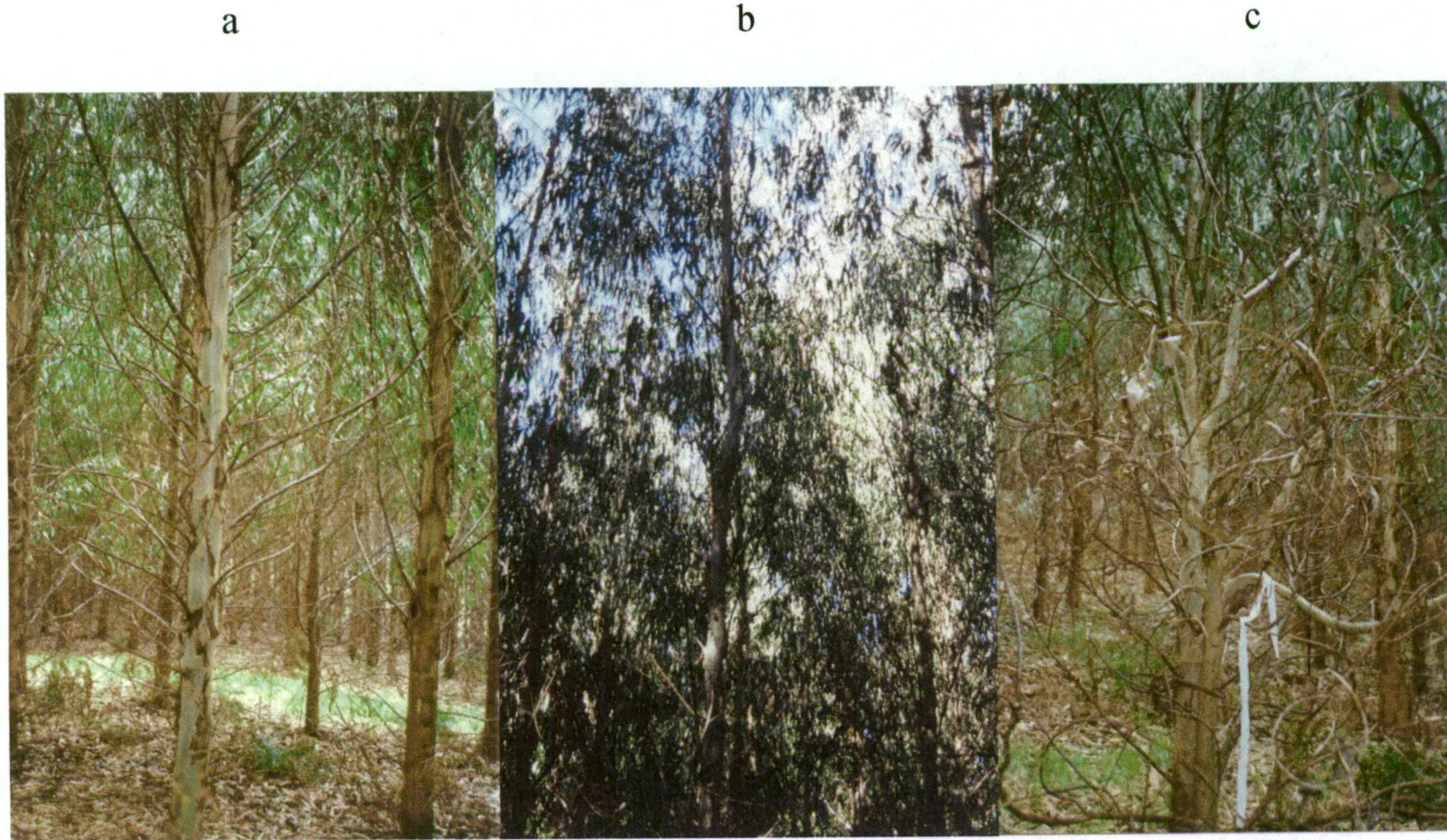


Plate 3.1: Trees from Gould's block with differing degree of deformity. a) straight tree, b) sinusoidal growth, but straight habit, c) leader dieback and severe distortion

3.2.3.2 Sampling of plant materials

Samples were taken from trees according to the severity of the symptoms (Plate 3.1 a-c):

- a) straight
- b) sinusoidal growth, but straight habit
- c) leader dieback and severe stem distortion

Samples of leaves, bark and wood of branches, roots <3 mm, and bark and wood of roots >3 mm were taken from one tree from each level of severity of symptoms. Root samples were taken from two additional straight and severely distorted trees. Root samples were taken from different parts of the root system during their excavation. Leaves, bark and wood were sampled from the top 3 m of the trees and taken from all branches. Only visually clean and healthy plant parts were sampled. The leaves and bark were wiped with detergent and rinsed twice with distilled water. The roots were brushed to remove any loose soil. For all wood samples the bark of the branch or root was first scrubbed with detergent and rinsed, and subsequently the bark was peeled off the wood. The samples were dried at 65°C and ground in a stainless steel mill with a 2 mm sieve size.

3.2.3.3 Excavation of root systems

The root systems were excavated after the trees had been felled at breast height. The topsoil was carefully removed to expose surface roots and to assess the extent of the root system. A trench was dug at the outer edge of the root system taking care not to cut through larger roots. The soil was removed from the root system by carefully raking it into the trench. This method allowed removal of the soil with minimum

damage to the root system, but no particular care was taken to preserve fine roots (<1mm diameter).

3.2.3.4 Soil and plant analysis

Loss on ignition, soil pH (1:5 soil/water) and EDTA – extractable Cu were determined using methods described in Rayment and Higginson (1992). The tissues and soils samples were digested in a H₂SO₄/H₂O₂ digestion (Lowther, 1980). The samples were oven dried at 65°C and 0.32 ± 0.02 g of each sample transferred into the digestion tube and weighed to four decimal places. After digestion the residue was diluted to 50 mL and filtered (Whatmann 42) into screw cap vials. The residue of the root and soil samples was washed into the filter paper, oven dried and ashed at 550°C in a muffle furnace for 4 h. The oven dry weight of the ash was determined.

The acetic acid and EDTA extracts were analysed for Cu and the digests for Cu, Mn, and Zn with Atomic Absorption Spectroscopy (SpectrAA-400, Varian Australia Pty Ltd, Mulgrave, Victoria).

The results for the root tissues was adjusted to the ash free content for soil contamination by the method of Misra (1994).

3.2.3.5 Statistical analysis

All results were analysed by Analysis of Variance using Genstat 5. The statistical model used was a fixed effect model (Model I) (Steel & Torrie, 1980)

3.3 Results

3.3.1 Stem deformity and nutrient concentrations

Table 3.1 shows the results of foliar analysis of trees from Gould's block comparing nutrient concentrations of straight and deformed trees two years after planting.

3.3.1.1 All straight trees vs. all deformed trees

The foliar concentrations of Cu and Zn were significantly lower in deformed than in straight trees (Table 3.1). The mean Cu concentration of all deformed trees (0.69 mg/kg) was less than a third of that of straight trees (2.26 mg/kg). The effect on Zn concentration was less pronounced and it was 22 % lower in deformed (10.7 mg/kg) than in straight trees (13.8 mg/kg). All other elements had greater foliar concentration in deformed compared to straight trees, but the difference was not significant for B, Fe and Mn.

3.3.1.2 Straight trees from plots with only straight trees vs straight trees from plots containing deformed trees

Cu and Zn were significantly lower in straight trees from plots with both straight and deformed trees than in those from plots with only straight trees (Table 3.1). The difference was especially strong for Cu where the concentration was almost halved from 2.61 to 1.36 mg/kg. Mn was significantly lower (812 mg/kg) in trees from mixed plots than in trees from plots with only straight trees (982 mg/kg). N, P and Mg had significantly higher concentrations in mixed plots.

Table 3.1: Foliar concentrations of 11 elements in straight and deformed trees two years after planting. a) Comparison of all straight and all deformed trees. b) Comparison between straight trees from plots with only straight trees (1), straight trees from plots with straight and deformed trees (2), deformed trees from plots with straight and deformed trees (3) and deformed trees from plots with only deformed trees (4) (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and n.s. = not significant)

a)	N (%)	P (%)	K (%)	Ca (%)	Mg (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	B (mg/kg)	Fe (mg/kg)	Al (mg/kg)
All straight trees	1.54	0.119	0.513	0.320	888	2.26	934	13.8	19.0	43.6	15.3
All deformed trees	1.72	0.144	0.643	0.355f	1037	0.69	1032	10.7	21.1	42.6	25.1
Significance	***	***	***	*	***	***	n.s.	***	n.s.	n.s.	***
b)	N (%)	P (%)	K (%)	Ca (%)	Mg (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	B (mg/kg)	Fe (mg/kg)	Al (mg/kg)
Straight trees from plots with only straight trees (1)	1.51	0.114	0.509	0.326	867	2.61	982	14.6	19.3	44.1	15.7
Straight trees from plots with straight and deformed trees (2)	1.61	0.132	0.523	0.305	943	1.36	812	11.6	18.2	42.3	14.1
Deformed trees from plots with straight and deformed trees (3)	1.73	0.142	0.638	0.359	1010	0.73	1040	10.2	20.4	43.2	24.1
Deformed trees from plots with only deformed trees (4)	1.69	0.147	0.653	0.347	1091	0.59	1016	11.8	22.6	41.5	27.2
Significance 1 vs 2	*	***	n.s.	n.s.	*	***	**	**	n.s.	n.s.	n.s.
Significance 2 vs 3	*	n.s.	**	n.s.	n.s.	**	**	*	n.s.	n.s.	***
Significance 3 vs 4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Significance 1 vs 4	**	***	***	n.s.	***	***	n.s.	n.s.	n.s.	n.s.	***

3.3.1.3 Straight vs. deformed trees in mixed plots

Cu and Zn were significantly lower in deformed trees than in straight trees and Cu concentration almost halved from 1.36 to 0.73 mg/kg (Table 3.1). N, K, Mn and Al were higher in deformed trees

3.3.1.4 Straight trees from plots with only straight trees vs. deformed trees from plots with only deformed trees

Cu concentrations were significantly lower in the deformed trees, with the concentrations less than 25% of those of straight trees (Table 3.1). N, P, K, Mg, and Al had significantly higher concentrations in deformed than in straight trees.

3.3.2 Effect of N and P fertilisation on foliar nutrient concentrations

3.3.2.1 After 6 months

Both N and P increased with higher N and P applied (Table 3.2). Cu was significantly lower with applied N and P. K, B, Fe and Al showed significant differences between different levels of N and P, but there was no consistent trend related to the amount of fertiliser applied.

3.3.2.2 After 2 years

Table 3.3 shows the effect of N and P application on the foliar nutrient concentrations of trees two years after planting. The data is taken from Table 3.1 and for plots with both straight and deformed trees the mean concentration was used. There were no significant interactions between N and P application and only the main effects are considered.

Table 3.2: Effect of N and P fertilisation on the foliar concentrations of 11 elements, six months after planting. (n=12) (Different letters in a column indicate significantly ($p<0.05$) different values)

	N (%)	P (%)	K (%)	Ca (%)	Mg (mg/kg)	
N1/P1	1.25 ^a	0.071 ^a	0.509 ^a	0.380	1228	
N2/P2	1.56 ^b	0.094 ^a	0.604 ^{a,b,c}	0.411	1163	
N3/P3	1.67 ^{b,c}	0.109 ^{a,b}	0.733 ^c	0.394	1236	
N4/P4	1.72 ^{b,c}	0.170 ^c	0.668 ^{b,c}	0.341	1168	
N5/P5	1.84 ^c	0.154 ^{b,c}	0.561 ^{a,b}	0.377	1219	
	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	B (mg/kg)	Fe (mg/kg)	Al (mg/kg)
N1/P1	2.07 ^b	584	12.1	15.3 ^b	40.7 ^a	17.0 ^{a,b}
N2/P2	1.51 ^a	658	12.8	10.3 ^a	48.0 ^{a,b}	20.6 ^{b,c}
N3/P3	1.49 ^a	529	12.3	14.1 ^b	55.9 ^b	20.6 ^{b,c}
N4/P4	1.49 ^a	624	10.2	11.0 ^a	40.2 ^a	13.6 ^a
N5/P5	1.43 ^a	465	13.2	11.5 ^a	49.2 ^a	22.0 ^c

Table 3.3: The main effects of a) N and b) P fertilisation on the foliar concentrations of 11 elements, two years after planting. The concentrations were taken from the data of Table 3.1. Were samples were taken from both deformed and straight trees in one plot the average concentration was used. Different letters in a column indicate significantly ($p < 0.05$) different values) There were no interactions between N and P and only main effects are presented

a)	N (%)	P (%)	K (%)	Ca (%)	Mg (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	B (mg/kg)	Fe (mg/kg)	Al (mg/kg)
N1	1.44 ^a	0.113	0.520	0.316	817 ^a	2.69 ^b	935	14.7	18.7	42.6	14.9
N2	1.54 ^{a,b}	0.120	0.543	0.341	875 ^{a,b}	2.58 ^b	1018	14.2	18.3	45.7	17.1
N3	1.59 ^{b,c}	0.125	0.566	0.328	922 ^{a,b,c}	1.96 ^{a,b}	894	12.9	19.2	43.7	15.8
N4	1.60 ^{b,c}	0.120	0.524	0.331	955 ^{b,c}	1.42 ^a	978	12.3	21.3	39.0	20.5
N5	1.67 ^c	0.137	0.560	0.330	1016 ^c	1.38 ^a	1020	12.8	20.5	45.5	20.4
b)	N (%)	P (%)	K (%)	Ca (%)	Mg (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	B (mg/kg)	Fe (mg/kg)	Al (mg/kg)
P1	1.47	0.109 ^a	0.498 ^a	0.311 ^a	866	2.88 ^b	920	14.7	17.7	38.8	12.3 ^a
P2	1.56	0.114 ^a	0.499 ^a	0.323 ^a	884	2.12 ^{a,b}	982	13.7	19.8	43.7	16.1 ^{a,b}
P3	1.58	0.121 ^{a,b}	0.545 ^{a,b}	0.328 ^a	890	2.06 ^{a,b}	980	12.6	19.9	43.6	17.2 ^{a,b}
P4	1.61	0.138 ^c	0.599 ^b	0.309 ^a	964	1.43 ^a	937	13.5	19.5	44.4	18.2 ^b
P5	1.61	0.133 ^{b,c}	0.571 ^b	0.374 ^b	982	1.55 ^a	1028	12.6	21.1	46.0	24.9 ^c

N concentration increased with each level of N application (Table 3.3). The concentration increased significantly with the application of more than 60 kg/ha N. Only 480 kg/ha N resulted in a significantly greater N concentration than 60 kg/ha N. Mg concentration showed a similar trend to N, but only at 240 kg/ha N or more was there a significant increase of Mg. Cu concentration was reduced by increased levels of N. There was a significant decrease of Cu concentration with the application of 240 and 480 kg/ha N compared to 0 and 60 kg/ha N.

P increased with P application up to 120 kg/ha (Table 3.3). P was significantly greater at 120 kg/ha P or more compared with 0 and 30 kg/ha applied P. The trend for K was identical to that of P. Ca (0.374 %) was significantly greater at 240 kg/ha P than at all other levels of P. Al increased with the level of P and a significant increase compared to no applied P occurred with 120 kg/ha P. There was a further significant increase in Al from 18.2 to 24.9 mg/kg with 240 kg/ha P. Cu decreased with increasing level of P up to 120 kg/ha. 120 kg/ha P or more resulted in significantly lower Cu than with no applied P.

3.3.3 Amelioration of stem deformity with micronutrient application

Table 3.4 shows results from the amelioration trial. Only the application of Cu alone or in combination with B resulted in a significant ($p < 0.05$) difference in DI after amelioration. Cu alone reduced the DI by 1.1 after 5 months and by 1.4 after 8 months. The effect of the combined treatment with B was less pronounced, reducing the DI by 0.8 after 5 months and by 0.9 after 8 months. Both these treatments also resulted in higher foliar concentrations of Cu compared to a reduction of foliar Cu in the control treatment. The concentrations of Mn was lower after 8 months, while the other elements showed a trend towards increasing concentrations.

Table 3.4: Results from the amelioration trial 1992/93. A) absolute values and b) differences in DI and foliar concentrations of Cu, Mn, Zn and B due to amelioration treatments. * indicates a significant ($p < 0.05$) difference in absolute value due to amelioration treatment. (Different letters in each date and column indicate significant differences between treatment effects ($p < 0.05$, $n = 10$))

a)		DI	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	B (mg/kg)
Before	Control	4.4	2.14	1284	16.4	20.6
	Complete mix	4.2	1.38	843	17.2	22.4
	B	4.9	1.77	1424	11.2	23.6
	Cu	4.9	1.63	1280	11.95	20.5
	Cu + B	4.1	1.36	1279	10.1	22.3
After 5 months	Control	4.2	1.36*	597*	13.5	17.2
	Complete mix	4.2	1.30	694	15.7	18.8
	B	4.4	1.54	509*	14.7	17.3*
	Cu	3.8*	2.10	524*	16.0	16.5
	Cu + B	3.3*	1.88	450*	16.3	15.8*
After 8 months	Control	4.4	2.36	843	17.2	22.4
	Complete mix	4.2	1.42	717*	15.1	20.8
	B	4.4	2.04	575*	20.4*	20.2
	Cu	3.5*	2.48	710*	19.4	21.0
	Cu + B	3.2*	2.23	811*	17.4	23.7
b)		DI	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	B (mg/kg)
After 5 months	Control	-0.2 ^a	-1.55 ^a	-581	-2.8	-3.38
	Complete mix	0 ^a	-0.09 ^b	-402	5.1	-0.43
	B	-0.5 ^{a,b}	-0.23 ^b	-915	3.5	-6.30
	Cu	-1.1 ^b	0.47 ^b	-755	4.1	-3.91
	Cu + B	-0.8 ^b	0.53 ^b	-828	6.2	-6.44
After 8 months	Control	0.0 ^a	-0.79	-334 ^a	0.9	1.83
	Complete mix	0.0 ^a	0.04	-378 ^a	4.4	1.59
	B	-0.5 ^{a,b}	0.27	-849 ^b	9.2	-3.37
	Cu	-1.4 ^c	0.85	-569 ^{a,b}	7.4	0.50
	Cu + B	-0.9 ^{b,c}	0.78	-467 ^a	7.3	1.38

3.3.4 Survey in 1996

3.3.4.1 *Soil*

The soil was strongly duplex and the depth of the sandy A-horizon determined if the soil was podsolised or if a true podsol developed. The A₁-horizon was an acid (pH 4.0-4.9), organic, loamy sand. The border between the A- and B-horizon was wavy and created a number of deep sand pockets (of about 80 cm), that alternated with an extremely shallow A-horizon. In the sand pockets the deeper sandy horizon developed a pronounced E-horizon indicating strong leaching of metals and organic matter. Above the B-horizon sandstone-like concretions in a number of colours formed in some cases (Figure 3.2 and Plate 3.2). The root distribution in the soil was influenced both by soil chemical and mechanical properties. Roots in the excavated root systems avoided the leached E-horizon and could not penetrate the concretions.

3.3.4.2 *Trees*

Trees in the trial showed a variety of symptoms, which ranged from sinusoidal growth to leader dieback and severe stem distortion (Plate 3.1 a-c). Many previously severely affected trees were now showing straight growth with a number of parallel leaders. In some cases whole plots were affected severely, but often the severity of the symptoms was very variable within a plot. The trees in the plantation showed a wide variety of growth rates. Severely deformed trees showed low growth rates, particularly where leader dieback was evident, as this was associated with the development of short internodes. Healthy trees had a high growth rate. The straight trees that were excavated grew in soil with a deeper A-horizon which contained a thick organic A₁-horizon, while deformed trees grew in soil with shallow top spoil.

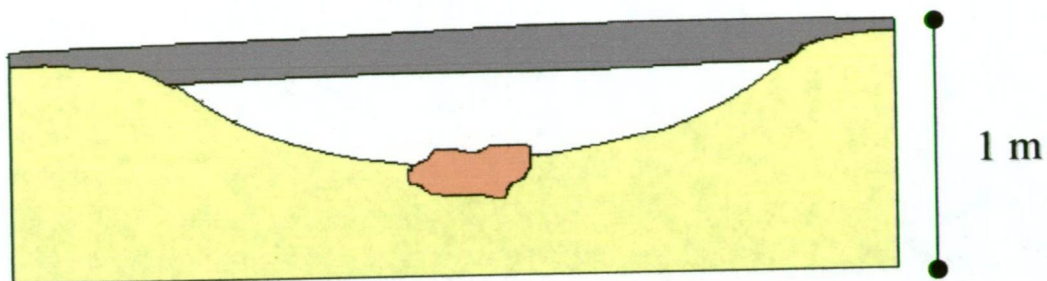


Figure 3.2: Diagram of the soil profile at Gould's block showing the wavy character of the subsoil. Yellow: B-horizon, Orange: Concretion, Dark grey: A-horizon, White: E-horizon



Plate 3.2: The border between the white E horizon on the left and the yellow B-horizon on the right. The tree in this picture is of Class II.

3.3.4.3. *Roots*

The root systems in areas of shallow topsoil were often stunted and displayed brown lesions. These lesions occurred as brown craters (Plate 3.3). Microscopic investigation of hand sections showed remnants of a lateral root in the middle of a lesion. The tissue was stained dark brown and the order of cell walls was disintegrated. Scars on big roots showed that the lesions could grow to considerable size and where the original epidermis had been sloughed off strong callus formation could be seen.



Plate 3.3: Roots from a Class III tree. Note the dark stained lesions and the lack of fine roots

Similar lesions affected the stems of some trees, but to a lesser extent. Stem lesions did not seem to be associated only with deformed trees, but also occurred on straight trees with a high growth rate. Small lesions occurred as blisters of the bark with the underlying tissue stained dark brown. Bigger lesions opened and the bark tissue contracted leaving an open crack.

3.3.4.4 Soil analysis

There were differences in soil characteristics between fertiliser treatments, but these were not related to the amount of fertiliser applied or the severity of stem deformities (Table 3.5). The results for duplicate fertiliser treatments showed differences up to 100%. The value of the differences of soil characteristics in duplicate plots were not correlated or related to the amount of fertiliser applied. The amount of Cu extracted by acetic acid was in the order of one percent of EDTA-extractable Cu.

Table 3.5: Deformity index and some soil characteristics of a range of fertiliser treatments. The two values are results from two plots which received the same fertiliser combinations

Treatment	Deformity Index	pH	Loss on ignition	EDTA – Cu (mg/kg)	Acetic acid – Cu (ppb)
N1/P1	0.1	4.5	13.0	0.77	1.9
	0.7	4.6	10.7	0.44	--
N2/P2	0.4	4.9	6.6	0.40	--
	1.0	4.6	8.1	0.40	7.5
N3/P3	0.6	4.9	6.9	0.38	5.6
	0.9	4.8	7.9	0.38	4.9
N3/P4	1.1	4.6	7.6	0.35	6.2
	2.3	4.4	12.5	0.56	6.2
N4/P3	1.8	4.6	8.3	0.48	4.1
	2.3	5.1	7.7	0.46	7.0
N4/P4	1.2	4.4	8.1	0.61	3.7
	1.2	4.5	8.5	0.40	3.4
N5/P5	3.7	4.3	10.2	0.80	3.3
	2.8	4.6	8.5	0.43	6.2

Table 3.6: Variation of a) EDTA – Cu and b) pH in several plots with differing fertiliser treatments, The results are from 8 samples taken from each plot in a 2 x 3.5 m grid as shown in Figure 3.1

a)	EDTA – Cu (mg/kg)							
Treatment	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
N1/P1	0.78	0.67	0.67	0.89	0.65	0.45	0.91	0.55
N3/P3	0.25	0.38	0.36	0.30	0.23	0.32	0.23	0.40
N4/P4	0.86	0.57	0.48	0.52	0.35	0.48	0.69	0.69
b)	pH							
Treatment	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
N3/P3	4.4	4.8	4.8	4.8	5.0	4.6	4.5	5.2
N4/P4	4.1	4.3	4.0	4.4	4.6	5.1	4.3	4.6
N5/P5	4.6	3.9	4.2	4.0	4.0	3.9	4.9	4.1

The variability of pH and EDTA-Cu of selected plots was considerable (Table 3.6). (The plots were selected to represent a range of fertiliser treatments as well as a range of pH and EDTA-Cu). The range of EDTA-Cu from some plots exceeded that of the total range of all plots measured (Table 3.5).

Soil from the root zone of deformed trees differed from that of straight trees (Table 3.7). The soil pH under deformed trees was 4.2 , which was significantly lower than the pH under straight trees (4.5). The difference in pH represents a doubling in the hydrogen ion concentration. Soil under deformed trees had a significantly higher LOI than that under straight trees (10.5 % and 8.3%, respectively). There was no significant difference between the soil Cu, Mn and Zn concentrations under both classes of trees, but Cu and Mn in the root zone of deformed trees was reduced to three quarters of that under straight trees .

Table 3.7: pH, loss on ignition and micronutrient concentrations of soil (0-10 cm) from the root zone of straight and severely deformed trees (see Figure 3.1 a and c) (* = $p < 0.05$, ** = $p < 0.01$) Values represent means of three samples.

	Straight trees	Severely deformed trees	l.s.d. ($p < 0.05$)
Soil pH	4.49	4.17	0.18 **
Loss on Ignition (%)	8.3	10.5	1.8 *
Soil Cu (mg/kg)	3.4	2.6	2.3
Soil Mn (mg/kg)	72.4	58.5	42.8
Soil Zn (mg/kg)	5.2	5.4	0.8

3.3.4.5 Plant analysis

Table 3.8 shows the distribution of micronutrients between different plant parts of three trees harvested in 1996. The distribution pattern in the tissues differed between elements. Cu and Zn showed much higher concentrations in the fine roots than in the leaves, while the Mn concentration was several times greater in the leaves than in the fine roots. All elements, apart from Cu, showed the lowest concentration in the branch wood, while Cu had similar concentrations in all aerial parts. The highest concentration of Zn was in the bark of branches.

Table 3.8: Mean Cu, Mn and Zn concentrations of plant parts taken from three trees of differing deformity classes

Plant part	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)
Wood	2.2	98	4.3
Bark	2.1	454	19.8
Leaves	2.1	845	9.7
Roots > 3 mm (wood)	1.0	44	11.5
Root < 3mm	5.9	135	16.9

Table 3.9: Cu, Mn and Zn concentrations of roots < 3 mm from three straight and three deformed trees . (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$)

Stem form	Cu (mg/kg)	l.s.d. _{0.05}	Mn (mg/kg)	l.s.d. _{0.05}	Zn (mg/kg)	l.s.d. _{0.05}
Straight	4.88	0.583	75.2	24.20	17.1	4.46
Deformed	2.89	***	94.4		10.1	**

Fine roots of straight trees had significantly ($p < 0.001$) higher Cu concentrations than those of deformed trees. The Cu concentration of straight trees (4.88 mg/kg) was almost 170% of that of deformed trees (2.89 mg/kg). Zn concentration showed a similar increase in roots of straight trees, but the significance level was only $p < 0.01$. Fine roots of deformed trees had a 25 % higher Mn concentration (94.4 mg/kg) than those of straight trees, but the difference was not significant due to variability of the Mn concentrations.

3.4 Discussion

3.4.1 Stem deformity and nutrient concentrations

The results presented above support the hypothesis of Turnbull *et al.* (1994) that the stem deformity observed at high levels of N and P application in the trial at Gould's Block is associated with fertiliser-induced Cu deficiency. However, they indicate a possible interaction with Zn deficiency on expression of symptoms. Application of high rates of N and P resulted in lower foliar Zn concentration after two years, although the difference was not significant. Turnbull *et al.* (1994) did not find a significant regression between Zn concentration and the severity of the syndrome, but reanalysis of their data showed that deformed trees as a group had lower Zn concentrations than straight trees. Deformed trees that grew in plots that contained also straight trees, had particularly low Zn concentrations. The results of Turnbull *et al.* (1994) appear to be the only ones that are used to indicate the adequate range of Zn concentration for *E. nitens* (Boardman *et al.*, 1997). It would not be appropriate to compare the Zn concentrations to the range established from the same site, but the Zn concentrations measured in deformed trees is in the marginal to deficient range reported for other Eucalyptus species (Dell *et al.*, 1995).

The differences in Zn concentration are not as pronounced as those for Cu, but the expression of Zn deficiency symptoms could well contribute to the severity of the deformities and the loss of upright habit. Short internodes, like those observed on severely deformed trees, have been associated with Zn deficiency (Bergmann, 1992) and are thought to be caused by a reduction in the synthesis of auxin in Zn deficient plants. Unfortunately the amelioration trial did not include a treatment of Zn alone or

in combination with Cu and it is therefore not possible to explore whether an increase in Zn concentration would have been involved in a lower DI.

The complete micronutrient treatment did not significantly improve the DI or to increase Cu concentrations. One of the possible explanations for these results is an antagonistic effect of another applied micronutrient. Unfortunately, it has not been possible to determine the actual composition of the complete micronutrient fertilizer that was applied in the amelioration treatment. Mn was likely part of the complete treatment and may have been the cause of this interference. The reduced branching of the root systems of deformed trees could easily interfere with Cu uptake. The lesions observed on these root systems are similar to those described for stems of *Malus domestica* suffering from internal bark necrosis (Eggert & Hayden, 1970), a disorder which was strongly associated with excess Mn (Hoyt, 1988). Al toxicity is another possibility as deformed trees showed a significant increase in Al concentration. Al toxicity is less likely to be a contributing factor as it affects mainly the root cap and its symptoms are short, stubby roots with a loss of ramification in the root system (Foy *et al.*, 1978). Although the affected root systems at Gould's Block showed a reduction of the number of fine roots, this was the result of lateral root dieback rather than inhibition of lateral root initiation. There also was no apparent inhibition of the extension of surviving roots. Either Mn or Al toxicity could have been caused by a decrease in the soil pH below 4.2 and are consistent with the lower soil pH measured in the root zone of deformed trees. Corroborating evidence that the effect is caused by Mn rather than Al toxicity comes from the plots which have both straight and deformed trees. Straight trees in these plots had a Cu concentration that was below the 1.4 mg/kg, the concentration at which deformities should have started, but their Mn

concentration was significantly lower than that of trees from plots with only straight trees. There was no significant difference in the Al concentration of straight and deformed trees in mixed plots.

3.4.2 Nutrient interactions

N and P application reduced the Cu concentration close to the critical level of 1.4 mg/kg suggested by Turnbull *et al.* (1994) after 6 months. The levels N and P at which Cu is reduced to this level were also close to those at which stem deformities are likely to appear (300 kg/ha N and 150 kg/ha P) (Charles Turnbull, personal communication). A reduction of Cu concentration with N application at a marginal supply of Cu has been reported for *T. aestivum* (Brennan, 1993) and forest tree species (van den Burg, 1983). A reduction of Cu concentration of several tree species due to P application has also been reported previously (Smilde, 1973). Saur *et al.* (1995) reported a reduction in the xylem sap concentration of Cu in *P. maritima* due to P application. It is therefore likely that the effect of N and P application on stem deformity occurred primarily due to induced Cu deficiency, as no other nutrient concentration was significantly reduced by their application. It is interesting to note that Al concentration showed an increase with applied P. This is contrary to previously reported results, where P application was used to lower toxic levels of Al due to the precipitation of either Al hydroxides or Al phosphates (Robson & Pitman, 1983).

3.4.3 Symptom expression

The high variability of the symptoms expressed in most treatment combinations is likely due to the interaction between a genetic variation in the susceptibility to stem deformity and the variable nature of the soil. The expression of the symptoms in *P.*

radiata shows strong genetic variation (Pederick *et al.*, 1984; Bail & Pederick, 1989). That this should be also true for *E. nitens* was indicated by the observation that straight and deformed trees were growing sometimes side by side. It was found during excavation that roots extended into the root zone of neighbouring trees and therefore they were likely to share the same soil. The expression of symptoms was patchy in nature with groups of straight and deformed trees in the same plots. This type of variation is more likely to be due to the variable nature of the soil. Of special interest is the wavy nature of the B-horizon, which severely restricted the effective root exploitation volume of some trees. The reduction of the root exploitation volume is one of the factors that may be interfering with uptake of Cu. The lower pH experienced may have exacerbated the problem by inhibiting root growth. The effect of a reduced root system would have been stronger for Cu than for nutrients like N and P, because Cu uptake relies more on root interception than either of these nutrients (see Table 2.1 from Bergmann, 1992). The amount of N and P present in the soil was also high, while the soil was low in Cu. The strong within plot differences in EDTA-extractable Cu indicates a high variability of available Cu in the soil. This is also likely to have contributed to the variable expression of symptoms.

Differences in soils under straight and deformed trees were found. The effect that the lower soil pH may have had on the extent of the root system has already previously been discussed. The lower soil pH was also likely to be the reason for the lower Cu concentration of soil under deformed trees. A lower pH results in less specific adsorption of Cu to soil particles (McBride, 1994) and increased leaching. On the other hand the amount of dissolved organic matter is reduced in a more acid soil, which may be the cause of the higher LOI of soil under deformed trees. The Cu bound

to this organic matter is not likely to be in a very plant available form, because the more available form would have been leached away. It has also been suggested that adsorption to root cell walls is the first step in Cu uptake (Kochian, 1993). If this step is necessary for Cu uptake to proceed, it may be reduced with the lower pH and increased protonation of the cell wall. The relative effect of soil pH on the adsorption by soil particles and cell walls would depend on the protonation of their respective adsorbing sites at a given pH.

3.4.4 Conclusions

Turnbull *et al.* (1994) already concluded that Cu deficiency was the probable cause of stem deformity in fertilised *E. nitens*. The results of this chapter strengthen that conclusion by the presentation of the results of the amelioration study. However, the results also show that other micronutrients, namely Mn and Zn, may be involved in the expression of the symptoms. It was possible to show that the expression of symptoms also depends on the soil and that the soil at the site studied is very variable. Furthermore it was shown that N and P application reduced the foliar Cu concentration of young *E. nitens* trees providing direct evidence that the Cu deficiency suspected at this site is indeed fertiliser induced.

The results presented in this chapter leave some questions that will be explored in the next chapter. Although Zn concentration was lower in deformed trees, there was no significant effect of N and P application on foliar Zn concentration. Application of N and P in a controlled environment would offer an opportunity to further explore, if there is a fertiliser induced Cu and Zn deficiency at this site. It was not possible to clearly identify mechanisms by which Cu deficiency was induced by high N and P

application. Low soil pH, high Mn levels and reduction in the size of root systems may have interacted. It was unfortunately for logistic reasons not possible to excavate the complete root systems of trees, while preserving all fine roots. Although there is limited application of their results to the field, pot experiments will allow the harvest of complete root systems to investigate if a reduction of root system size is associated with high N and P application and a reduction of Cu concentration in the plants.

Chapter 4: Fertiliser-induced Cu and Zn deficiency in *E. nitens*: greenhouse experiments using seedlings, clones and a test plant

4.1 Introduction

4.1.1 Background

In Chapter 3 it was shown that application of N and P significantly reduced the foliar Cu concentration of *E. nitens*, but not of the other micronutrients measured, although Zn showed a trend to lower concentration at high N and P. Trees displaying deformities had lower foliar Cu and Zn concentrations than straight trees while several nutrients, including Mn, had higher concentrations in deformed trees than in straight trees. These results suggested that Zn deficiency interacts with the induced Cu deficiency as a cause of deformities observed in *E. nitens* grown by Turnbull *et al.* (1994) and that high levels of Mn may have been a contributing factor, either by direct inhibition of Cu uptake or by impeding root growth.

In order to investigate and understand the occurrence and effect of micronutrient deficiencies, it is necessary to undertake designed experiments in controlled environments to exclude a number of interacting environmental factors present in the field. Due to the long life cycle of forest trees, and in the case of *E. nitens* the very slow growth of small seedlings, such experiments can be very time consuming. To overcome this problem it is desirable to use a fast growing annual species as a test plant which is sensitive to the nutrient studied. A further advantage of using a well

studied agricultural species as a test plant in nutritional studies is that deficient and adequate ranges have been firmly established in the literature, while the experimental basis for such ranges is much smaller for forest trees. For example, the adequate range of nutrients for *E. nitens* presented in Boardman *et al.* (1997) is based on only one experiment (Turnbull *et al.*, 1994).

4.1.2 *L. esculentum* as a test plant

Avena sativa, *T. aestivum*, *Z. mays*, *Medicago sativa*, *Lactuca sativa*, *Daucus carota*, *Allium cepa*, *L. esculentum*, and *Nicotiana tabacum* have been described as good ‘indicator’ plants for Cu deficiency (Robson & Reuter, 1981; Reuther & Labanauskas, 1966). Of these *A. cepa* and *L. esculentum* are sensitive to Zn as well as Cu deficiency (Chapman, 1966). Only *L. esculentum* is also sensitive to Mn excess (Labanauskas, 1966). *L. esculentum* was chosen as the most suitable plant to investigate the micronutrients suspected of causing the stem deformities in *E. nitens* reported by Turnbull *et al.* (1994). Concentrations of the nutrients studied reported in the literature as deficient, adequate and toxic for *L. esculentum* are summarised in Table 4.1.

4.1.3 Objectives of the experiments

The first experiment was used to induce copper deficiency in *E. nitens* and *L. esculentum*. *L. esculentum* acted as a test plant for *E. nitens*. The effect of two soil types associated with straight and deformed trees in the field, and two levels of N, P, Cu, Mn and Zn on the growth and micronutrient concentration of eucalypt seedlings were studied. The soil used was from a trial in which application of fertiliser-induced Cu deficiency led to unacceptable levels of stem deformity (Turnbull *et al.*, 1994).

Table 4.1: Concentration ranges of Cu and Zn for *L. esculentum* reported in the literature

Nutrient	Culture	Plant part	Concentration range (mg/kg)					Reference
			deficient	marginal	adequate	high	toxic	
Cu	Field	Shoots			5-15			Huett <i>et al.</i> , 1997
Cu	Greenhouse	Leaves			3.10-12.30			Reuther & Labanauskas, 1966
Cu	Solution	Leaves	1		10			Wallihan & Bradford, 1977
Zn	Field	Shoots	<20	20-30	30-200			Huett <i>et al.</i> , 1997
Zn	Soil	Shoots			50-150		250-500	Huett <i>et al.</i> , 1997
Zn	Greenhouse	Shoots	<25					Lingle, <i>et al.</i> , 1958
Zn	Greenhouse	Shoots			50-150	250-380	>450	Boawn & Rasmussen, 1971
Zn	Solution	Leaves	23		57			Wallihan & Bradford, 1977

Mn and Zn were identified as possible contributing factors to the stem deformities at the site (Chapter 3).

A second trial was used to further investigate the suitability of *L. esculentum* as a test plant for Cu deficiency in *E. nitens*. The factors in this trial are two levels of Cu, two soils and two clones of *E. nitens*. One soil was that associated with deformed trees used in the first experiment, the other was from a second trial where stem deformities had also occurred.

The experiments test the following hypotheses:

- Soil associated with deformed trees in the field will induce Cu and/or Zn deficiency in *E. nitens* and *L. esculentum* seedlings.
- Application of the high rate of N and P will lead to a reduction in Cu and Zn concentration in the seedlings
- Uptake of Cu and Zn and the response to N and P application differs between plants grown in the two soils in Experiment 1.
- Application of Mn will lead to interference with root growth and/or Cu uptake.
- Application of Cu and Zn will ameliorate deficiencies and increase growth rate.
- *L. esculentum* will act as an indicator plant for Cu and Zn deficiencies in *E. nitens* in both experiments.

4.2 Materials and methods

4.2.1 Experimental design

Two pot trials were undertaken in a greenhouse in 1996/97 and 1998/99. The first trial (Experiment 1) had a 2^6 factorial design with the following factors (Table 4.2): Two soils and two levels of added N (130 mg/kg and 600 mg/kg), P (260 mg/kg and 1100 mg/kg), Cu (0 mg/kg and 24 mg/kg), Mn (0 mg/kg and 16 mg/kg) and Zn (0 mg/kg and 10 mg/kg). The pots were arranged in a randomised complete block design with two blocks. The second trial (Experiment 2) had a 2^3 factorial design with the following factors (Table 4.2): Two soils, two clones of *E. nitens*, two levels of added Cu (0 mg/kg and 10 mg/kg). The pots were arranged in a randomised complete block design with four blocks.

Table 4.2: Levels of nutrient treatments (in mg per kg air dry soil) for Experiment 1 and 2.

a	Nutrient	Low treatment (mg/kg air dry soil)	High treatment (mg/kg air dry soil)
Experiment 1	N	130	600
	P	260	1100
	Cu	0	24
	Mn	0	16
	Zn	0	10
Experiment 2	Cu	0	10

4.2.2 Soil and plant material

4.2.2.1 Experiment 1

The two soils for the experiment were taken from the topsoil (0-10 cm) of a fertiliser trial where stem deformity had occurred due to fertiliser-induced Cu deficiency (Turnbull *et al.*, 1994). At three locations in the trial, soil was sampled from plots where groups of both deformed and straight trees occurred. Soil 1 was taken from amongst groups of deformed trees and Soil 2 from amongst groups of straight trees. Both soils were organic, loamy sand. Some properties of the soils are shown in Table 4.3. The soil was sieved through a 5 mm sieve, mixed well and air dried at 30°C. The plant material used was seedlings of *L. esculentum* var. Grosse Liesse and *E. nitens*. (seed lot EXT 230, ex North Forest Products (NFP), Burnie).

4.2.2.2 Experiment 2

The two soils used in this experiment were taken from two trials in which stem deformities associated with application of fertiliser had occurred. One trial (Gould's) is that described by Turnbull *et al.* (1994) and the soil was from similar sites to Soil 1 in Experiment 1. The other (Penna) is a trial studying weed competition on the establishment of *E. globulus* with the application of up to 800 kg N per ha. From each trial 30 kg of topsoil (0-10 cm) were removed from approximately 1 m² at four sites throughout each of the trials. Large roots and weeds were removed from the soil. The soil was air dried and sieved through a 5 mm plastic sieve. The four bags of soil from each trial were bulked and thoroughly mixed. The Cu, Mn and Zn concentrations of soil from each sample site were determined (Table 4.3). The plant material was seedlings of *L. esculentum* var. Grosse Liesse and two *E. nitens* clones (E 533 and E 534, ex NFP).

Table 4.3: Some chemical characteristics of the soils used in Experiments 1 and 2.

Organic carbon was estimated by Loss on Ignition (LOI); total nutrient concentrations were determined using acid digestion.

Experiment	Soil	Site	LOI (%)	pH	Total Cu (mg/kg)	Total Mn (mg/kg)	Total Zn (mg/kg)
Experiment 1	Soil 1	1	8.5	4.5	2.0	6.5	6.8
		2	8.8	4.7	2.3	3.7	6.6
		3	8.5	4.2	3.0	5.8	11.2
	Soil 2	1	8.8	5.1	2.6	24.3	8.8
		2	10.3	4.7	3.5	4.3	7.3
		3	10.9	4.6	3.8	8.7	18.1
Experiment 2	Gould's	1			1.7	42.6	2.9
		2			3.0	36.4	5.5
		3			2.6	45.7	6.5
		4			1.9	35.8	4.2
	Penna	1			1.7	219	10.7
		2			1.2	222	8.1
		3			1.3	217	6.2
		4			1.6	265	12.8

Table 4.4: Type and amount of nutrients (in mg) added to pots prior to planting in Experiments 1 and 2. Analytical reagents were used throughout the experiments.

b	Nutrient	low treatment (mg/pot)	high treatment (mg/pot)
Experiment 1	N as $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	2191	10114
	P as $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	2116	8952
	Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0	189
	Mn as $\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0	98
	Zn as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0	88
	K as KCl	1551	1551
	Mg as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	820	820
	B as H_3BO_3	113	113
Experiment 2	N as urea	1826	1826
	P as $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	2278	2278
	Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0	138
	Mn as $\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0	0
	Zn as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0	0
	K as KCl	2714	2714
	Mg as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1435	1435
	B as H_3BO_3	198	198

4.2.3 Preparation of pots

Before use the pots were soaked in acid detergent and rinsed three times with deionised water. 2 kg of the air dried soil was used per pot for Experiment 1 and 3.25 kg of air dried soil per pot for Experiment 2. After weighing, the soil for each pot was moistened to reduce the strong water repellence of the dry soil. The nutrient salts for each treatment combination (Table 4.4) were mixed with acid washed sand (<0.5 mm) and ground finely in a ceramic mortar. Additional sand was ground in the mortar to

clean it between treatments (altogether 140 mL of sand were used per pot). The nutrients and the sand were spread over the moistened soil and mixed well. The soil was filled into black, plastic pots of 17.5 cm (Experiment1) and 20 cm (Experiment 2) diameter and compressed to give a dry bulk density of 1.0 g cm^{-3} . After planting the surface of the pot was covered with 5 mm acid washed sand (diameter between 0.5 and 2 mm). Pots were checked daily, and watered when the sand layer was dry.

4.2.4 Plant propagation and management

4.2.4.1 Experiment 1

Twelve seeds of *L. esculentum* per pot were planted (Figure 4.1). The pots were watered with Fongarid solution to inhibit damping off and covered with cling wrap until seedling emergence. Emergence of each seedling was recorded and the median day of emergence used as the reference point for plant measurements. Plant height was measured weekly from two weeks after emergence until the final harvest. After the first true leaves emerged, the tallest and the smallest plants in each position were thinned out. The remaining four plants were sprayed to incipient runoff with 100 mg/kg Mo solution.

After the harvest of the *L. esculentum* shoots, the pots were air dried to completely desiccate the roots. Subsequently the pots were sprayed with water at frequent intervals to rehydrate the soil.

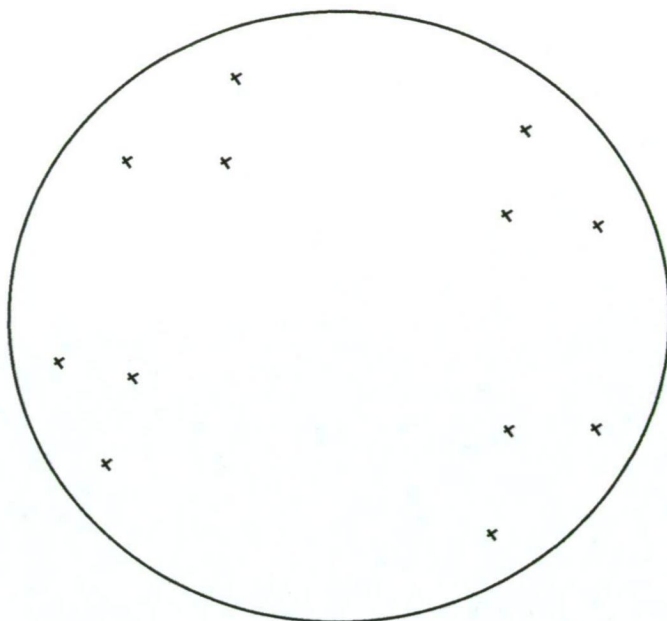


Figure 4.1: Arrangement of seeds of *L. esculentum* var. *Grosse Liesse* for Experiment 1



Plate 4.1: Sand tray with *E. nitens* seedlings prior to transplanting.

The seeds of *E. nitens* were osmotically primed for five days in 2 % KNO_3 solution. The seeds were then washed and transferred to a sand tray using a pasteur pipette . This method allowed the seeds to be spread rapidly (1000 seeds/h) on the sand trays and no thinning was necessary (Plate 4.1).

When most seedlings had expanded their first true leaves, they were transplanted into the pots from the tomato experiment. Five seedlings were planted in each pot (Plate 4.2) and thinned after four weeks, leaving the largest seedling. Measurements of plant height commenced when plants had expanded three leaf pairs and were taken at the same intervals as for *L. esculentum* seedlings. Height was measured from the soil to the tip of the last leaf pair.



Plate 4.2: *E. nitens* seedlings at the start of measurements.

4.2.5 Plant harvest

4.2.5.1 Experiment 1

The seedlings were grown until they started to compete strongly in the pots. They were then harvested to reduce the influence of effects other than nutrients on the growth rate. The *L. esculentum* seedlings were harvested five weeks after germination. The shoots were cut at the soil surface. *E. nitens* seedlings were harvested nine weeks after measurements started. The shoots were cut at the soil surface and divided into leaves and stems. The plants were washed with acid detergent (Sonneveld & van Dijk, 1982), rinsed three times with deionised water, drained and dried at 60°C to constant weight.

No roots of *L. esculentum* were harvested. For *E. nitens*, the pots containing the roots were kept in the greenhouse after harvest of the shoots and processed after all shoots were harvested. Harvested roots showed no visible signs of deterioration. The sand covering the soil was removed with a brush and the contents of the pot were tipped into a 5 mm sieve. The soil was carefully teased apart using a plastic comb and the root systems removed. Loose roots were removed using the plastic comb. The soil was then passed through the 5 mm sieve and the retained roots were collected. The root systems were dried for 15-30 min and as much of the soil adhering to the roots as possible was removed by shaking. This soil was retained to represent the rhizosphere soil. The roots were soaked briefly and rinsed in deionised water to remove the bulk of any remaining soil. The roots were then floated in deionised water and organic debris was removed from the roots with forceps. Any remaining soil contamination of the roots was estimated by the method of Misra (1994). Clean roots were drained and dried at 60°C.

4.2.5.2 Experiment 2

Shoots of *L. esculentum* were harvested after four weeks. New growth of the *E. nitens* clones was harvested one week afterwards and again eight weeks later. The plant material was treated in the same manner as in Experiment 1.

4.2.6 Plant analysis

The dried plant tissue was ground to < 2 mm using a stainless steel Wiley Mill and redried at 60°C. 750 mg of ground tissue were digested using a modified HNO₃/H₂O₂ digestion (Xing & Venemann, 1998) and analysed for Cu and Zn.

For the digestion, the 750 (+/- 50) mg of ground plant tissue were weighed to four decimal places into dry digestion tubes. 7.5 mL of concentrated HNO₃ were added with thorough mixing. The mixture was left for 1-2 h and shaken occasionally. The mixture was slowly heated to 120° C in an aluminium heating block. During heating, the mixture had to be shaken, and the heating stopped when the reaction got too vigorous. Tear drop stoppers were inserted on the tubes after foaming subsided and the digest was mainly liquid. The digestion mixture was refluxed at approximately 120° C for 1 h. The digest was cooled in the block until the temperature was below 70° C and then 4 mL H₂O₂ were added in two portions (the reaction is very vigorous at first). The mixture was refluxed at 120° C for 1 h. During refluxing, the fumes should condensate half way up the tube. The block was switched off and 35 mL deionised water were added when it had cooled to below 100° C. When cool, the mixture was made up to 50 mL with deionised water and mixed with a vortex mixer. The residue in the digest was left to settle for 24 h. The clear, pale yellow digest was poured into an

acid washed container without disturbing the residue. This allowed the supernatant to be removed without the possibility of contamination by centrifuge containers. Initial tests were conducted to ensure that the digest stayed mixed during the settling period. 40-45 mL of digest were generally recovered.

The digests were directly aspirated and analysed by Atomic Absorption Spectroscopy (SpectrAA-400, Varian Australia Pty Ltd, Mulgrave, Victoria).

4.2.7 Soil analysis

Loss on ignition and soil pH (1:5 soil/water) was measured as described in Raymond and Higgins (1992).

The soil analysis for Cu and Zn used the same method as for plant materials, but 1 g of air dried soil was used. 10 mL HNO₃ were added instead of 7.5 mL and the length of the first boiling step was 2 h.

4.2.8 Ratios of nutrients between soil, roots and shoots

The ratios of measured micronutrient concentration in soil, roots and shoots was used (in this and the following chapters) to identify where in the soil/plant system the induced micronutrient deficiency occurred. For example a decrease in the root : soil ratio would indicate that the entry into the root was inhibited. Although this method ignores factors like the soil availability of nutrients, it provides an indication of factors on which later more detailed research can be conducted.

4.2.9 Statistical analysis

The data was analysed by Analysis of Variance using Genstat 5. The statistical model used was a fixed effect model (Model I) (Steel & Torrie, 1980).

No transformation of data (except for the Cu concentration of roots, which was log transformed for Analysis of Variance) was necessary. Each data set was tested for normality and constant variance.

4.3 Results

There were significant interactions between all treatments. Only the results of interactions will be presented here. The significant interactions presented are always the highest order between specific factors.

4.3.1 Effect of soil and nutrient applications on the growth of *E. nitens* seedlings in Experiment 1

4.3.1.1 *Effects of interactions between soil and nutrient applications*

The growth of all plant parts measured showed a significant interaction between soil and N and P application. The effect of the treatments was similar for height and dry mass (Table 4.5). With low N or P the plants grown in Soil 2 were significantly smaller than those at the same level of N and P in Soil 1. However, plants grown in Soil 2 responded more strongly to N and P application than those grown in Soil 1. A large increase in dry mass was observed and shoot dry mass increased about fivefold while that of roots increased threefold. The difference between Soil 1 and 2 was especially pronounced when both high N and high P were applied. High P application resulted in a reduction of growth in Soil 1 with high N. In Soil 2, application of high N and high P led to a significant increase in growth. With low N the shoot : root ratio was higher in Soil 1 than in Soil 2. The difference in shoot : root ratio between plants in Soil 1 (5.7) and in Soil 2 (4.6) was significant with low N and high P. The ratio was significantly higher with high N than with low N. There was no significant difference in ratio between treatments with high N apart from Soil 2 with low P which was higher (10.6) than all others (7.4 – 8.1).

Table 4.5: The effect of N and P application on the two soils on height, dry mass of different plant parts and shoot : root ratio of *E. nitens* seedlings and total dry mass of *L. esculentum* in Experiment 1. (n = 16) Different letters in each row indicate significant ($p < 0.05$) difference.

	Soil 1				Soil 2			
	N130 P260	N130 P1100	N600 P260	N600 P1100	N130 P260	N130 P1100	N600 P260	N600 P1100
<i>E. nitens</i>								
Height (mm)	284 ^b	319 ^c	357 ^d	336 ^c	233 ^a	282 ^b	320 ^c	358 ^d
Stem dry mass (g)	2.1 ^c	2.8 ^d	4.1 ^{c,f}	3.7 ^c	0.8 ^a	1.5 ^b	3.0 ^d	4.4 ^f
Leaf dry mass (g)	6.2 ^c	8.2 ^d	11.7 ^{f,g}	10.6 ^f	2.8 ^a	4.2 ^b	9.4 ^c	12.2 ^g
Root dry mass (g)	1.6 ^c	2.0 ^d	2.1 ^d	1.9 ^{c,d}	0.8 ^a	1.3 ^{b,c}	1.2 ^b	2.3 ^e
Shoot dry mass (g)	8.2 ^c	11.1 ^d	15.8 ^{e,f}	14.2 ^e	3.6 ^a	5.7 ^b	12.4 ^d	16.6 ^f
Total dry mass (g)	9.9 ^c	13.0 ^d	17.9 ^{e,f}	16.2 ^e	4.4 ^a	7.0 ^b	13.6 ^d	18.9 ^f
Shoot : root ratio	5.3 ^{a,b}	5.7 ^b	7.4 ^c	8.1 ^c	4.7 ^a	4.6 ^a	10.6 ^d	7.6 ^c
<i>L. esculentum</i>								
Dry mass (g)	3.8 ^{b,c}	3.3 ^b	3.7 ^{b,c}	1.8 ^a	4.8 ^d	4.9 ^d	4.1 ^c	4.9 ^d

There was a significant ($p < 0.05$) interaction between soil and Mn application for the dry mass of roots, leaves, shoots and whole plants. The trends for the different tissues were identical. Plants grown in Soil 1 showed a significant increase in dry mass due to Mn application, while plants grown in Soil 2 did not show a significant effect of applied Mn (Table 4.6).

Table 4.6: The response of dry mass of *E. nitens* seedlings to Mn application on the two soils in Experiment 1 ($n = 32$). Different letters in each row indicate significant ($p < 0.05$) differences.

	Soil 1		Soil 2	
	Mn0	Mn16	Mn0	Mn16
Root dry mass (g)	1.8 ^b	2.1 ^c	1.4 ^a	1.4 ^a
Stem dry mass (g)	3.0 ^b	3.3 ^b	2.5 ^a	2.3 ^a
Leaf dry mass (g)	8.7 ^b	9.6 ^c	7.4 ^a	6.9 ^a
Shoot dry mass (g)	11.7 ^b	12.9 ^c	9.9 ^a	9.3 ^a
Total dry mass (g)	13.5 ^b	15.0 ^c	11.2 ^a	10.7 ^a

4.3.1.2 Effects of interactions between nutrients

There was a significant ($p < 0.05$) interaction between N, P and Cu application on plant height and stem dry mass (Table 4.7). At low N, there was a significant increase in height with high P, but no significant response to applied Cu (Table 4.7). At high N, high P alone caused a trend towards lower plant height (from 345 to 328 mm) but this was not statistically significant. A positive response (332 to 366 mm) to high P application occurred only with the application of Cu. Significant increases in stem dry mass occurred for high N or high P alone with or without applied Cu (Table 4.7). A further significant increase with the combination of high N and high P occurred only with the application of Cu.

Table 4.7: The response of height and stem dry mass of *E. nitens* seedlings to N, P and Cu application in Experiment 1 ($n = 16$). Different letters for each measurement indicate significant ($p < 0.05$) differences.

		N130		N600	
		P260	P1100	P260	P1100
Height (mm)	Cu0	255 ^a	293 ^b	345 ^c	328 ^c
	Cu24	262 ^a	308 ^b	332 ^c	366 ^d
Stem dry mass (g)	Cu0	1.30 ^a	2.02 ^b	3.66 ^c	3.60 ^c
	Cu24	1.57 ^a	2.29 ^b	3.40 ^c	4.44 ^d

There was a significant ($p < 0.05$) interaction between P and Mn application on dry mass. There was no significant response to applied Mn at low P, but at high P, there was a significant increase of dry mass (Table 4.8). Root, stem and total dry mass were significantly greater with high P at both levels of applied Mn. For leaf and shoot dry mass, a significant increase at high P occurred only with applied Mn.

Table 4.8: The response of dry mass of *E. nitens* seedlings to Mn application at two levels of P in Experiment 1 ($n = 32$). Different letters in each row indicate significant ($p < 0.05$) differences.

	P260		P1100	
	Mn0	Mn16	Mn0	Mn16
Stem dry mass (g)	2.6 ^a	2.4 ^a	2.9 ^b	3.2 ^c
Leaf dry mass (g)	7.7 ^{a,b}	7.3 ^a	8.4 ^b	9.2 ^c
Shoot dry mass (g)	10.3 ^{a,b}	9.7 ^a	11.3 ^b	12.5 ^c
Total dry mass (g)	11.7 ^a	11.1 ^a	13.0 ^c	14.5 ^d

There was a significant ($p < 0.05$) interaction between N, P and Mn application on root dry mass (Table 4.9). Root dry mass was not significantly affected by applied Mn at either low N and/or low P. When both N and P were high, a significant increase of root dry mass from 1.7 to 2.4 g occurred in response to applied Mn.

Table 4.9: The response height and stem dry mass of *E. nitens* seedlings to N, P and Mn application in Experiment 1 ($n = 16$). Different letters for each measurement indicate significant ($p < 0.05$) differences.

		N130		N600	
		P260	P1100	P260	P1100
Root dry mass (g)	Mn0	1.2 ^a	1.2 ^a	1.7 ^b	1.7 ^b
	Mn16	1.6 ^b	1.6 ^b	1.8 ^b	2.4 ^c

There was a significant interaction between P, Cu and Zn application on height ($p < 0.05$) and dry mass ($p < 0.001$). Plants grown at high P and applied Cu had significantly greater height than at low P (Table 4.10). When Cu and Zn was applied, height was significantly greater than with all other treatment combinations. The pattern for dry mass was similar to that of height, but dry mass at low P and no Cu was significantly lower than all treatments at high P.

Table 4.10: The effect of P, Cu and Zn application on height, dry mass, leaf and root Cu concentration of *E. nitens* seedlings in Experiment 1.

Different letters in each row indicate significant ($p < 0.05$) differences

	P260				P1100			
	Cu0 Zn0	Cu0 Zn10	Cu24 Zn0	Cu24 Zn10	Cu0 Zn0	Cu0 Zn10	Cu24 Zn0	Cu24 Zn10
Height (mm)	292 ^a	307 ^{a,b}	296 ^{a,b}	298 ^{a,b}	313 ^{b,c}	308 ^{a,b,c}	326 ^c	348 ^d
Stem dry mass (g)	2.2 ^a	2.8 ^c	2.7 ^{b,c}	2.3 ^{a,b}	3.0 ^c	2.6 ^{b,c}	3.0 ^c	3.7 ^d
Leaf dry mass (g)	6.7 ^a	8.4 ^b	7.6 ^{a,b}	7.3 ^{a,b}	8.6 ^b	8.0 ^b	8.4 ^b	10.2 ^c
Root dry mass (g)	1.4 ^a	1.5 ^{a,b,c}	1.5 ^{a,b}	1.3 ^a	1.9 ^{c,d}	1.7 ^{a,b,c}	1.7 ^{b,c}	2.2 ^d
Shoot dry mass (g)	8.9 ^a	11.2 ^c	10.3 ^{a,b,c}	9.5 ^{a,b}	11.6 ^c	10.6 ^{b,c}	11.4 ^c	14.0 ^d
Total dry mass (g)	10.3 ^a	12.9 ^{b,c}	11.8 ^{a,b}	10.9 ^a	13.5 ^b	12.3 ^b	13.3 ^b	16.2 ^c
Leaf Cu concentration (mg/kg)	3.4 ^b	3.1 ^b	8.2 ^d	9.1 ^e	2.0 ^a	2.3 ^a	7.6 ^d	6.5 ^c
Root Cu concentration (mg/kg)	7.1 ^{a,b}	7.3 ^b	25.9 ^d	29.7 ^e	6.3 ^a	6.9 ^{a,b}	22.5 ^c	29.9 ^e

4.3.2 Effects of soil and nutrient applications on the micronutrient concentrations in leaves and roots of *E. nitens* seedlings in Experiment 1

4.3.2.1 Effect of interactions on Cu concentration

There was a significant interaction between P, Cu and Zn application on the Cu concentration in leaves and roots. Without applied Cu, there was a significant reduction in Cu concentration of both tissues at high P and no significant effect of applied Zn (Table 4.10). With applied Cu, applied Zn resulted in significantly lower Cu in leaves at high P (7.6 to 6.5 mg/kg) and significantly higher Cu at low P (8.2 to 9.1 mg/kg). For roots, Cu concentration increased with applied Zn at both levels of P. A significant reduction in Cu due to high P (25.9 to 22.5 mg/kg) occurred only without applied Zn.

There was a significant interaction between soil, N, P and Cu application on the Cu concentration in leaves and roots (Table 4.11). Plants with applied Cu had higher Cu concentrations for all treatment combinations. There were no significant differences in Cu concentration due to level of N or P in Soil 1 without applied Cu. With applied Cu, plants in Soil 1 showed a significant increase in leaf Cu concentration with N. Without applied Cu, plants grown in Soil 2 had significantly lower leaf Cu at high P at both levels of N and root Cu only at high N. High N reduced leaf and root Cu only with high P. With applied Cu, both leaf and root Cu concentration were significantly reduced by high P only with high N. The highest concentration in leaves (13.1 mg/kg) and roots (58.9 mg/kg) occurred in Soil 2 with applied Cu at high N and low P. These concentrations were 2.3 – 3.5 times higher for roots and 1.5 – 2.3 times higher for leaves than other treatments with applied Cu.

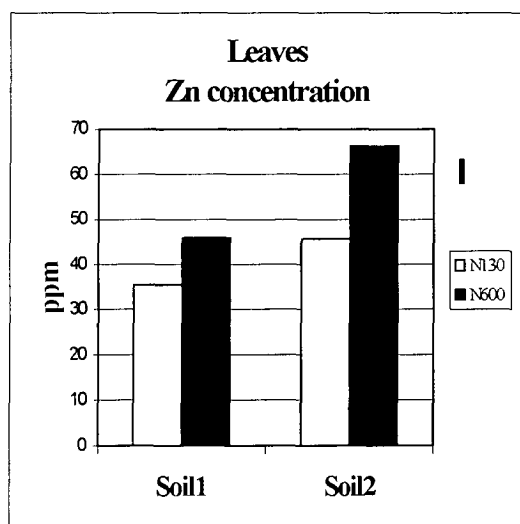
Table 4.11: The Cu concentration of leaves and roots of *E. nitens* seedlings in response to N, P and Cu application on two soils in Experiment 1 (n = 8). The data for the root concentration was log transformed for Analysis of Variance. Different letters for each plant part indicate significant ($p < 0.05$) differences.

			N130		N600	
			P260	P1100	P260	P1100
Cu concentration of leaves (mg/kg)	Soil 1	Cu0	1.6 ^a	1.5 ^a	1.4 ^a	1.4 ^a
		Cu24	6.1 ^{d,c}	5.6 ^{c,d}	7.5 ^{f,g}	7.3 ^f
	Soil 2	Cu0	5.2 ^{c,d}	3.4 ^b	4.8 ^c	2.0 ^a
		Cu24	7.9 ^{f,g}	6.9 ^{e,f}	13.1 ^h	8.6 ^g
Cu concentration of roots (mg/kg)	Soil 1	Cu0	6.5 ^{b,c}	6.1 ^b	4.6 ^a	4.8 ^a
		Cu24	19.4 ^{e,f}	17.4 ^c	21.7 ^{f,g}	19.7 ^{e,f}
	Soil 2	Cu0	9.6 ^d	8.9 ^d	9.2 ^d	7.2 ^c
		Cu24	23.1 ^g	22.4 ^{f,g}	58.9 ^h	26.0 ^g

4.3.2.2 Effect of interactions on the Zn concentration

There were significant interactions between Soil and applied N, and Soil and applied P on the Zn concentration of leaves. Zn concentrations were higher in Soil 2 than in Soil 1. In both soils, high N increased Zn (Figure 4.2 a). In Soil 1 this increase was by 30% from 35 to 46 mg/kg and in Soil 2 by 45% from 46 to 66 mg/kg. In Soil 2, high P reduced Zn by 27% from 65 to 47 mg/kg (Figure 4.2 b). There was no significant difference due to P in Soil 1.

a



b

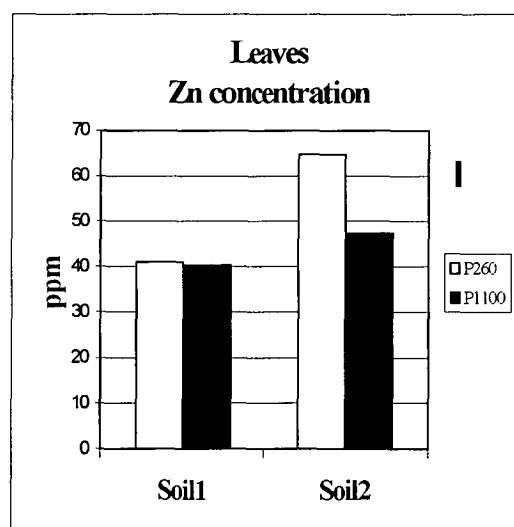
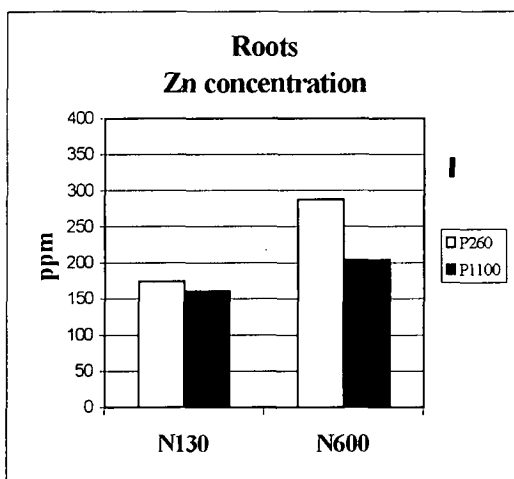


Figure 4.2: The response of Zn concentration of leaves of *E. nitens* seedlings to a) N and b) P application on two soils in Experiment 1 (n=32). The vertical bar represents the l.s.d._{0.05} = 5.9 mg/kg

There were significant interactions between applied N and P, and applied N and Zn on the Zn concentration of roots. Zn concentration was higher at high N at both levels of P or Zn. A significant reduction from 287 to 205 mg/kg due to level of P occurred only at high N (Figure 4.3 a). The increase in Zn with applied Zn was more strongly expressed at high N (143 to 349 mg/kg) than at low N (112 to 223 mg/kg) (Figure 4.3 b).

a



b

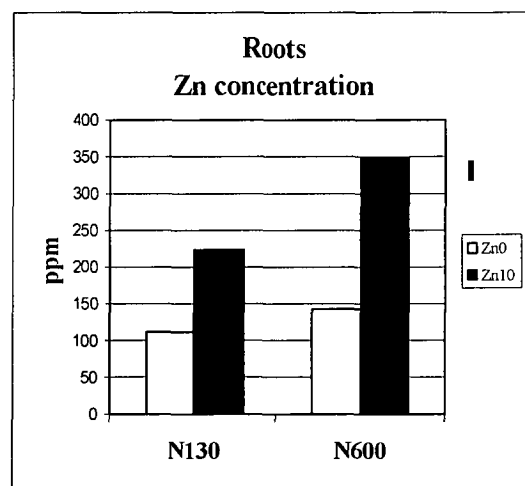


Figure 4.3: The response of Zn concentration of *E. nitens* roots to a) P and b) Zn application at two levels of N in Experiment 1 (n =32). The vertical bar represents the l.s.d._{0.05} = 29.7 mg/kg

4.3.2.3 Nutrient ratios between soil, root and leaves

The ratio of concentrations between roots and soil was an order of magnitude higher for Zn than for Cu (Table 4.12). The root : leaf ratio was moderately higher for Zn than for Cu. Plants grown in Soil 2 had a significantly greater root : soil of Cu than those in Soil 1, while the ratio was higher in Soil 1 for Zn. The root : leaf of both

micronutrients was significantly greater in plants grown in Soil 1. High N significantly increased the root : soil ratio of Zn from 17.6 to 23.6. High P significantly reduced the root : soil of both Cu and Zn. The root : soil of Cu was reduced by more than 50% from 3.14 to 1.36 with applied Cu. Applied Mn and Zn had no significant effect on the ratios except for a significant increase of the root : leaf ratio of Zn with applied Zn.

Table 4.12: Main effects of soil and N,P, Cu, Mn and Zn application on the ratios of Cu and Zn concentration between root and soil, and root and leaves of *E. nitens* in Experiment 1 (N = 64) Numbers in bold indicate a significantly higher value (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

<i>Nutrient</i>	Ratio	Soil1	Soil 2	N 130	N 600	P 260	P 1100
Cu	Root : soil	1.89	2.60***	2.24	2.26	2.43**	2.07
	Root : leaf	3.45*	3.14	3.27	3.32	3.20	3.38
Zn	Root : soil	22.6	18.6	17.6	23.6***	23.5***	17.7
	Root : leaf	4.5	3.9	4.1	4.3	4.3	4.1
<i>Nutrient</i>	Ratio	Cu 0	Cu 24	Mn 0	Mn 16	Zn 0	Zn 10
Cu	Root : soil	3.14***	1.36	2.20	2.30	2.23	2.26
	Root : leaf	3.20	3.39	3.24	3.34	3.29	3.29
Zn	Root : soil	20.3	20.9	19.8	21.5	20.3	21.0
	Root : leaf	4.3	4.1	4.1	4.3	3.0	5.4***

4.3.3 *L. esculentum* as a test plant

4.3.3.1 Plant growth

L. esculentum did not show the same growth response to Soil and nutrient applications as *E. nitens* (Table 4.5 for Experiment 1 and Table 4.13 for Experiment 2). In Experiment 1 growth of *L. esculentum* was strongly depressed in Soil 1. There was a particularly pronounced difference in growth in Experiment 2, where *L. esculentum* showed very poor growth in soil from Gould's (*E. nitens* grew better in that soil than in the Penna soil). Both plants showed little direct effect of Cu application on plant growth

Table 4.13 Response of dry mass, Cu and Zn concentrations of two *E. nitens* harvests (*E. nitens* 1 and *E. nitens* 2) and one of *L. esculentum* from Experiment 2 to soil and Cu application. For *E. nitens* only new growth was harvested, while the whole shoots of *L. esculentum* was harvested. The first *E. nitens* harvest was taken at the same time as the *L. esculentum* seedlings. The second harvest is subsequent growth.

Variable	Plant material	Gould's		Penna	
		Cu0	Cu10	Cu0	Cu10
Shoot dry mass (g)	<i>E. nitens</i> 1	7.1 ^b	7.6 ^b	0.7 ^a	0.8 ^a
	<i>E. nitens</i> 2	37.8 ^b	40.0 ^b	11.8 ^a	11.3 ^a
	<i>L. esculentum</i>	5.2 ^a	5.1 ^a	23.7 ^b	26.8 ^b
Cu concentration (mg/kg)	<i>E. nitens</i> 1	2.2 ^a	8.9 ^c	4.6 ^b	5.4 ^b
	<i>E. nitens</i> 2	1.2 ^a	5.4 ^c	3.1 ^b	6.3 ^d
	<i>L. esculentum</i>	2.0 ^a	9.2 ^c	2.7 ^a	7.7 ^b
Zn concentration (mg/kg)	<i>E. nitens</i> 1	38.9 ^c	39.0 ^c	33.7 ^b	29.8 ^a
	<i>E. nitens</i> 2	24.4 ^a	22.5 ^a	29.1 ^b	27.2 ^b
	<i>L. esculentum</i>	84.7 ^b	76.0 ^{a,b}	95.9 ^b	72.1 ^a

4.3.3.2 Cu and Zn concentration

It was not possible to analyse the nutrient concentrations of *L. esculentum* in Experiment 1 statistically because of an interaction of all factors. This higher order is impossible to analyse in a meaningful way and lower order interactions will be influenced by it. However, it is possible to compare the main effects of the treatments on the concentrations of *E. nitens* and *L. esculentum* seedlings to get an indication if the two plants would respond in a similar fashion to nutrient application and soil type. They showed a very similar response to soil and N and P application (Table 4.14). The only difference is that *E. nitens* showed an increase in Zn concentration from 40.6 to 56.1 mg/kg due to high N. This positive response of Zn concentration to N is absent in *L. esculentum*. *L. esculentum* showed responses of Cu concentration to Mn and Zn application and of Zn concentration to Cu application that did not occur in *E. nitens*. Cu concentration increased due to Mn application from 12.4 to 13.4 mg/kg and due to Zn application from 12.6 to 13.3 mg/kg. Cu application on the other hand reduced Zn concentration in *L. esculentum* from 153 to 130 mg/kg

In experiment 2 both species responded to applied Cu with a strong increase in Cu concentration (Table 4.13). Also the effect of soil on both plants was similar with lower concentration in the Penna soil without Cu application. Like in Experiment 1, the Zn concentration of *L. esculentum* was reduced by applied Cu and *E. nitens* did not show this response.

Table 4.14: Main effects of Soil and N, P, Cu, Mn and Zn application on Cu and Zn concentration in leaves of *E. nitens* seedlings and whole shoots of *L. esculentum* in Experiment 1 (n = 64). Numbers in bold indicate a significantly higher value (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

Nutrient	Plant tissue	Soil1	Soil 2	N 130	N 600	P 260	P 1100
Cu (mg/kg)	<i>E. nitens</i> leaves	4.1	6.5***	4.8	5.8***	6.0***	4.6
	<i>L. esculentum</i> Whole shoots	12.0	13.9***	12.6	13.2**	13.7***	12.2
Zn (mg/kg)	<i>E. nitens</i> leaves	40.7	56.0***	40.6	56.1***	52.9***	43.8
	<i>L. esculentum</i> Whole shoots	117	164***	143	139	160***	122
		Cu 0	Cu 24	Mn 0	Mn 16	Zn 0	Zn 10
Cu (mg/kg)	<i>E. nitens</i> leaves	2.7	7.9***	5.3	5.3	5.3	5.3
	<i>L. esculentum</i> Whole shoots	4.2	21.7***	12.4	13.4***	12.6	13.3***
Zn (mg/kg)	<i>E. nitens</i> leaves	46.7	50.0	49.4	47.3	42.7	54.0***
	<i>L. esculentum</i> Whole shoots	153***	130	141	142	102	180***

4.4 Discussion

The research in this chapter aimed to answer three main questions:

- 1) Do the soils supporting straight (Soil 2) and deformed (Soil 1) trees differ in the micronutrient supply to *E. nitens* and as a consequence, the growth response of the seedling to N and P application?
- 2) How do applications of N, P, Cu, Mn and Zn affect growth and Cu and Zn concentrations of *E. nitens*?
- 3) Can *L. esculentum* be used as a test plant for detecting Cu and Zn deficiencies in *E. nitens*?

4.4.1 Differences between soils in Experiment 1

Plants grown in Soil 2 had higher micronutrient concentrations than those grown in Soil 1. This can be explained by the higher total micronutrient concentration in Soil 2 than in Soil 1 at the start of the experiment. This higher concentration may be associated with the higher pH of Soil 2, which would result in greater adsorption and precipitation of Cu and Zn on soil solids. Cu and Zn would therefore be less mobile in Soil 2. Soil 1 may have been subject to some leaching of Cu and Zn from the topsoil, because its pH is very low. The leaching would have been particularly effective as only the top 10 cm of the soil were collected for the experiments. The higher organic matter content of Soil 2 (as indicated by the loss on ignition) is a further soil characteristic that could be responsible for the higher micronutrient content of Soil 2. Soil fractionation studies have found a large fraction of soil Cu and Zn in the organic matter fraction (McLaren & Crawford, 1973 a; Shuman, 1979), particularly in acid soils

(Sims, 1986). This fraction has been identified as the major pool available for plant uptake (McLaren & Crawford, 1973 a; Sims, 1986).

The higher root : soil ratio of Cu in Soil 2 indicates that the higher soil concentration was not the only reason for an increased uptake of Cu in that soil. The lower concentration in the roots relative to Soil 1 may have partly been a dilution effect as plants grown in Soil 1 had a higher root dry mass than those in Soil 2. Another aspect of the reduced Cu uptake from Soil 1 may have been its lower pH. Generally, availability of the total Cu present in soil increases with a lower pH due to reduced adsorption to ligands and an increase in the free ion activity (Sanders, 1982). Nonetheless, Alva & Chen (1995) showed a reduction of Cu concentration with a reduction of pH from 6.5 to 4.5 in citrus seedlings grown in nutrient solutions with high Cu concentrations. They explained the reduction with inhibition of Cu uptake by competition of protons for adsorption and/or absorption sites. The inhibition did not occur in solutions with a low Cu concentration, but the pH of Soil 1 was lower than that used in their experiment. The higher proton concentration in Soil 1 may have resulted in competition at a low Cu concentration. Another reason could be reduced mobility of Cu in the soil due to precipitation of dissolved organic matter, as discussed below.

Zn showed a lower root : soil ratio in Soil 2. The increased accumulation of Zn is probably due to the lower pH of the soil, which would effect its availability strongly (Msaki & Calvet, 1990). The effect of pH on Zn is stronger than that on Cu as dissolved organic matter, the main ligand for Cu in solution, precipitates at the low pH. Sanders (1983) reported a drop of the total Cu concentration in the soil solution

from 0.70 to 0.38 μM when the pH of a CaOH_2 -amended soil dropped from 6.6 to 4.75. This drop was accompanied by a reduction of the concentration of soluble Carbon from 75 to 35 μM . In the same experiment the soil solution concentration of Zn increased from 0.24 to 2.5 μM . Another possible explanation is a less efficient uptake mechanism of Zn at the higher soil concentration. Other nutrients have been shown to express multiphasic uptake kinetics with more efficient uptake at low soil concentration (Nissen, 1994). This explanation may be problematic, because the higher soil concentration does not necessarily mean a higher soil solution concentration and the uptake mechanisms respond to the soil solution concentration.

For both soils, the dry mass of plant parts was lowest when both N and P were applied at the low rate, indicating an insufficient supply of N and P, particularly in Soil 2. Thus the results in the pot experiment did not agree with those in the field. There, the unfertilised control had a high growth rate and there was no significant response to the application of N and P (Turnbull *et al.*, 1994). This difference between the field and the pot study may have been the result of removal of N and P by *E. nitens* trees during the five years growth prior to the collection of soil for the experiment. Also the soil was collected from the top 10 cm of the soil and some of the applied fertiliser may have been leached into a lower soil horizon. Therefore, the soil used in the experiment may have had a lower N and P content than that present when the young trees in the plantation were growing. The positive response of Soil 2 to high N and high P combined was likely due to the better Cu supply in that soil, since there was a positive interaction between N, P and Cu application on tree growth.

4.4.2 Effect of nutrient applications on plant growth and micronutrient concentration

An increase of height and stem dry mass of *E. nitens* with P application at high N occurred only when Cu was applied. The application of Cu showed no significant effect when N and/or P were low. These results show that without applied Cu uptake was sufficient to support growth at low N and P, but inadequate for an increase in growth rate at high N and P. A similar positive interaction between N and Cu application was found for *T. aestivum*. There was no improvement of grain yield by either nutrient applied alone, but an increase in yield when both were applied together (Chaudhry & Loneragan, 1970; Singh & Swarup, 1982). The need for a balance between N and Cu application can be explained by the essential role of Cu as part of enzyme systems (Marschner, 1995). In order for it to fulfil this role it relies on a sufficient N nutrition, because the synthesis of proteins is strongly related to the amount of N present in the plant. A particular aspect of the N, P and Cu interaction is indicated by research of Warren & Adams (2002). They showed that high P application to *P. pinaster* results in the storage of N in protein form as Rubisco rather than as soluble amino acids. Rubisco also contains Cu (Loneragen, 1981) and an accumulation of this protein may lead to reduced physiological availability of Cu for other enzymes essential for optimal growth. When Cu is applied, there will be enough free Cu present for the plant to achieve its full potential. A similar increase in shoot growth in combination with high N and P was not indicated for the other micronutrients. An increase in shoot growth due to Zn application occurred only at high P when Cu was applied as well. It can be concluded from these results that Cu becomes limiting after the application of high rates of N and P, and that Zn becomes only limiting when Cu is also applied.

There were interesting effects of nutrient application on the shoot : root ratio. An increase of the ratio due to the application of high N was expected (see Cannell & Dewar, 1994; Wilson, 1988). This may be one of the reasons that high N induces Cu deficiency, because Cu supply relies strongly on soil exploration by roots. An increase in shoot : root ratio with lower Mn application has also been reported previously (Wilson, 1988). On the other hand, the significant reduction in shoot : root ratio with high P application at high N in Soil 2 and the absence of a response in Soil 1 was unexpected, as the application of higher P should have the same effect as high N (Cannell & Dewar, 1994; Wilson, 1988). High P application with high N in Soil 2 resulted in a reduction of Cu concentration, which was similar to the Cu concentration in of plants in Soil 1. The low Cu concentration may have inhibited increased growth of shoots at high P more than that of roots as part of the Cu in the leaves may have been bound to surplus Rubisco (see above) reducing its physiological availability in the leaves. This would lead to a decrease in shoot : root ratio with high P at lower Cu concentration, an indication that an increase of shoot : root ratio due to the application of N and P relies on a high foliar Cu concentration (see also discussion in Chapter 6).

In combination with high N and P, Mn application had a positive effect on root growth in *E. nitens*. Mn also had no significant main effect on Cu concentration in *E. nitens*. The only significant reduction in Cu concentration with applied Mn occurred when Cu was applied as well and therefore had no effect on causing Cu deficiency. It is possible that the rate at which Mn was applied was insufficient to induce Mn toxicity. On the other hand these results may indicate, that Mn toxicity is not involved in the stem deformities found by Turnbull et al. (1994). The soils used for the experiments had lower Mn concentrations than those sampled in Chapter 3 (Table 4.3 and 3.7) and no

lesions were found on roots during the collection of soil for the experiments in Chapter 4. It is therefore quite possible that the lesions and also Mn toxicity are a local phenomenon.

E. nitens leaves in this study showed a decrease of Cu concentration at high N without applied Cu, and an increase of Cu concentration at high N with applied Cu similar to that found previously in *T. aestivum* (Singh & Swarup, 1982) and forest trees (van den Burg, 1983). The decrease of Cu concentration at high N occurred mainly in Soil 2 and can be at least partly explained by a dilution effect due to the increased dry mass. Another factor is the higher shoot : root ratio of plants at high N as has been suggested for *P. radiata* (Olykan & Adams, 1995). Cu is not a very mobile nutrient in the soil and its uptake depends significantly on the soil volume exploited by the root system. The smaller relative size of the root system may therefore result in a reduced supply of Cu to the shoots. The increase in Cu concentration at high N was not associated with a reduction of the root Cu concentration and the root : leaf concentration ratio was not affected by N. The increase was not due to an improvement in the root to shoot transport of Cu. An improvement of Cu uptake by the plants is also indicated by the strong increase in root Cu concentration when Cu was applied to Soil 2 and only N was high. Nitrate was used as the source of N in this experiment and therefore it is unlikely that there was an increase in Cu availability due to acidification of the root zone. It is more likely that the high N treatment resulted in increased synthesis of nitrogenous compounds that may be involved in the uptake of Cu. This mechanism of increased Cu uptake with high N has been suggested previously for *T. aestivum* (Singh & Swarup, 1982) and other plants (Gladstone *et al.*, 1975).

A significant reduction of Cu concentration with high P occurred only in Soil 2 and was at least partly due to a dilution effect, because seedlings responded strongly to high P application in that soil. High P exacerbated the effect of high N without applied Cu and it almost completely negated the increase of Cu concentration with high N and applied Cu in that soil. The effect of soil on reduction of Cu concentration by high P differed from that found by Smilde (1973), who reported a stronger effect of P on foliar Cu concentration in soil with a lower pH. In that study the difference in soil pH was achieved by liming. The difference between the results of Smilde (1973) and the results presented in this thesis may be due to the inhibition of Cu uptake with low P in the limed soil. At the high soil pH, there may not have been reduction due high P application, because Cu uptake was already limited by the soil pH. Cu concentration of cuttings in the limed soil at low P was at a similar level to that of cuttings with high P regardless of soil pH (Smilde, 1973).

Reduced Zn concentration due to P application was only found in combination with treatments that resulted in high Zn concentration at low P (Soil 2 and high N). This is contrary to previous findings with *G. hirsutum* in which low Zn concentration in the plant aggravated the effect of P application by interfering with the control of P uptake and translocation (Marschner & Cakmak, 1986). This interference led to the accumulation of high amounts of P in Zn deficient plants enhancing the effect of P application and even the expression of P toxicity symptoms (Loneragan *et al.*, 1979; Webb & Loneragan, 1988). P application was also found to affect the Zn translocation in the plant (Warnock, 1970). In the present study P application did not affect the Zn leaf : root ratio of the seedlings.

The effect of P application on Cu and Zn concentration in this study occurred during the uptake from the soil, since the root : soil ratio of Cu and Zn concentration was lower at high P. The P application rate of 1100 mg/kg was higher than in other investigations of P interaction with micronutrients which generally applied less than 500 mg/kg P (see for example Cakmak & Marschner, 1986, 1987; Shukla & Singh, 1979; Smilde, 1973). It is therefore likely that the inhibition was due to the formation of insoluble phosphates in the soil (Parker, 1981; Barak & Helmke, 1993). Nonetheless, the inhibition by P did not lower the micronutrient concentrations to deficient levels.

With applied Cu, the Cu concentrations in *E. nitens* increased from 0.9 - 7.1 mg/kg to 3.3 - 15.1 mg/kg. The range of the Cu concentrations of *E. nitens* seedlings in this study was greater than that (1.8 – 13.9 mg/kg) reported for other forest trees (van den Burg, 1983). Van den Burg's trial was conducted with a very acid, Cu deficient soil that contained only 0.8-1.3 mg/kg total Cu, and up to 20 mg/kg Cu were applied. The higher Cu concentration in the trees grown without applied Cu may have been due to species differences. The appearance of Cu deficiency symptoms on the seedlings indicate that they need higher Cu concentrations than *E. nitens* in which no symptoms of Cu deficiency were observed. There are two other possible reasons that trees in Van den Burg's trial had higher Cu concentrations without applied Cu. There may have been considerable reserves in the planting stock, as 1 year old nursery stock was used, and no precautions to limit uptake of Cu in the nursery were mentioned in the methods. The trees also showed a slow growth rate and the experiment was conducted over a long time, which may have allowed the trees to accumulate Cu from the Cu-deficient soil.

4.4.3 *L. esculentum* as a test plant

The Cu concentration of *L. esculentum* was higher than that of *E. nitens*, but without applied Cu it was below levels considered adequate for *L. esculentum* (Huett *et al.*, 1997). Cu application raised the Cu concentration in *L. esculentum* to levels above the adequate range of 5 – 15 mg/kg (Huett *et al.*, 1997). On the other hand the Zn concentration of *L. esculentum* was in the adequate range of 50 – 150 mg/kg (Boawn & Rasmussen, 1971; Huett *et al.*, 1997) with and without Zn application. No deficiency symptoms that could be linked to the application of Cu or Zn were present on the plants. These results show that *L. esculentum* grown in soil associated with stem deformities in *E. nitens* accumulate marginal to insufficient concentrations of Cu, but had sufficient Zn to support growth.

Cu concentration of *L. esculentum* showed similar responses to soil and nutrient application as that of *E. nitens*. This shows that *L. esculentum* can act as a good indicator plant for induced Cu deficiency in *E. nitens*. The value of < 5 mg/kg for *L. esculentum* (Huett *et al.*, 1997) proved to be more useful than 1.4 mg/kg for eucalypts in predicting response to Cu application in pot trials. In the Penna soil *E. nitens* concentrations without Cu application were above 3 mg/kg indicating sufficient Cu supply, but stem deformities in the field responded to Cu application. The concentrations found in the greenhouse were also not in accordance with lower concentrations measured in the field (unpublished data). In the same soil Cu concentrations of *L. esculentum* were 2.7 mg/kg, well below the level where deficiency is expected. In Soil 2 of Experiment 1 *L. esculentum* also had concentrations which indicated Cu deficiency. In the field, this soil was associated with straight trees at intermediate levels of N and P. However, at the highest N and P

applications, no difference in stem deformity due to soil could be observed. Therefore, also that soil could have benefited from Cu fertilisation, when very high rates of N and P are applied.

4.4.4 Conclusions

The results in this chapter did not show a clear effect of Mn and Zn application on the growth and Cu concentration of *E. nitens* seedlings. This may have been due to an insufficient application rate or interference by the strong effect of soil and N and P application. In Chapter 5 an experiment will be conducted, that will use additional rates of Mn and Zn and only one soil and one rate of N and P. This experiment will attempt to show if there is an interaction between the three micronutrients. This experiment will also run for a longer time to investigate if the positive response of growth to Cu application at high N and P can be maintained.

The results in this chapter indicate N as the primary cause of the fertiliser-induced Cu deficiency at Gould's plantation. In Chapter 6 an experiment will be conducted to identify where the induced deficiency occurs in the soil/plant system. One of the mechanisms that will be studied is the inhibiting effect of N application on senescence of leaves and the remobilisation of Cu from the senescent leaves. This effect has not only been well documented for *T. aestivum* (Hill *et al.*, 1978, 1979 a,b; Loneragan *et al.*, 1980), but there was some evidence in Experiment 1 in this chapter, that high N reduced senescence of leaves and inhibited withdrawal of nutrients from the leaves (Plate 4.3). Unfortunately, this effect was only noted after part of the seedlings had been harvested and quantitative analysis was not possible.

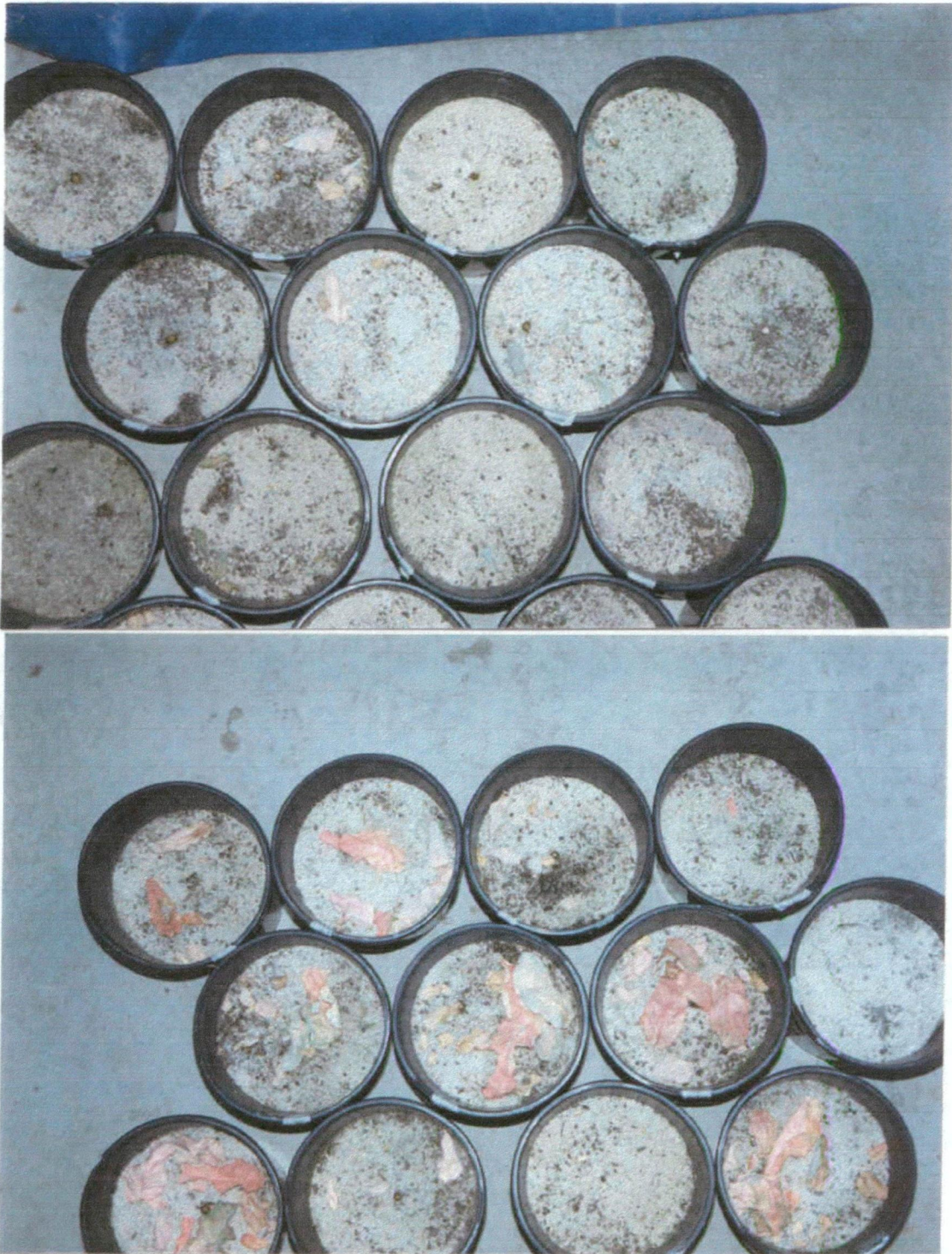


Plate 4.3: Difference in the senescence of leaves of *E. nitens* seedlings with high (above) and low (below) N. These pictures were taken after the tops of the plants had been harvested. Note, that plants with high N had not only fewer senescent leaves, but many leaves also did not show the red colour associated with removal of nutrients.

Chapter 5: Effect of Cu, Mn and Zn application on growth, micronutrient concentrations and stem form of *E. nitens* seedlings

5.1 Introduction

5.1.1 Background

Stem deformities in fertilised plantation trees were first reported in Australia for *P. radiata* (Ruiter, 1969) and Cu deficiency was suspected as the cause of the symptoms. Subsequent studies collated by Downes & Turvey (1992a) showed that Cu deficiency was the cause of the deformity in a number of cases. Affected trees had reduced foliar Cu concentrations and reduced lignification of the stem wood. The symptoms could be alleviated by the application of Cu fertiliser. Similar deformities which relate to application of N and P fertilisers have been described in *E. nitens* and Cu deficiency has been reported as the likely cause (Turnbull *et al.*, 1994). Deformed *E. nitens* had also lower foliar Zn concentrations than straight trees (see Chapter 3) and it is possible that Zn deficiency exacerbated the expression of symptoms. Loss of apical dominance and rosetting of terminal branches have been described as symptoms of Zn deficiency (Bergmann, 1992). The site at which the stem deformity was first described in *P. radiata* also had a history of Zn deficiency (Ruiter, 1969).

5.1.2 The control of stem form in trees

Form of trees is ultimately the result of internal and external forces working on the stem. When a stem is not moving, following Newton's Laws the sum of the forces and

the sum of the torques affecting it have to equal zero. External forces working on the tree are the effects of gravity and wind load; the internal forces are growth stresses in the stem. The form of the tree in equilibrium is determined by its genetic potential and the environment.

5.1.2.1 Gravitropism

Higher plants maintain form in a certain 'equilibrium position' termed the 'gravitropic set-point angle (GSA)' (Firn & Digby, 1997). The vertical leader is a special case of the GSA. Only the case of the vertical leader is considered here. Displacement of the leader from the GSA results in differential growth on the two sides of the organ, which return it to the equilibrium position. Epinasty, stress of bending and gravity have been postulated as the forces triggering this response, but the majority of evidence attributes the main role to gravity (Wilson, 1973). In herbaceous plants the change in the direction of the stem is the result of differential growth rates on both sides of the stem. For example, when hypocotyls of *Cucumis sativus* were placed in a horizontal position the expansion of the upper surface ceased, while the expansion rate of the lower surface increased two- to three-fold (Cosgrove, 1990). Woody plants, both gymnosperms and angiosperms, produce reaction wood to provide the motive force for the gravitropic response to a displacement of the main axis from the vertical. Gymnosperms produce compression wood on the underside of the disturbed stem, while in angiosperms, tension wood is formed on the upper side of the stem (Scurfield, 1973; Wilson & Archer, 1977).

5.1.2.2 Growth stresses and reaction wood

During the growth of a tree the outer layers show a decrease in longitudinal extension compared to the central core. This leads to the development of growth stresses in the stem as the core is under compression and the circumference under tension. The anatomy of reaction wood differs from that of normal wood. Compression wood contains a large number of heavily lignified tracheids, while tension wood has reduced conducting tissue and increased numbers of fibres (Wilson & Archer, 1977). The tracheids of compression wood contain a thick lignified (S_2) layer in their cell wall, while the fibres of tension wood are often termed 'gelatinous' due to a layer in their cell walls containing a low lignin concentration and a high content of cellulose (Scurfield, 1973). The anatomical changes in the reaction wood influence the balance of these stresses around the circumference of the stem and therefore regulate the form of the tree. During their maturation these tissues show longitudinal extension (compression wood) or shrinkage (tension wood) relative to the opposite side of the stem resulting in a net upward bending of the leaning stem (Kubler, 1987).

Currently two models describe how the growth stresses are produced during the maturation of the tissue. According to the lignin swelling model, the swelling of lignin during its biosynthesis leads to changes in the cells perpendicular to the average direction of cellulose microfibrils in the cell wall. In tension wood with a small microfibril angle to the cell axis, this leads to radial swelling of the cell wall and longitudinal shrinkage, while in compression wood with a large microfibril angle longitudinal extension is the result. Criticism of this model is based mainly on the absence of increased lignification in tension wood. In an alternative model, the growth stress is created by tension developing in the maturing microfibrils. Lignification

interferes with this process leading to negative tension (ie. compression) in gymnosperm reaction wood (Kubler, 1987).

5.1.3 Cu, Mn and Zn deficiency as causes for stem deformities

Deficiencies in micronutrients are likely to influence stem form through two processes. Firstly, the deficiency may inhibit lignification, which will lead to soft wood and potentially a loss of gravitropic response. Secondly, the deficiency may interfere with the hormonal control of gravitropism.

5.1.3.1 Lignification

Loss or reduction of lignification due to Cu deficiency has been reported for herbaceous and woody plants. The affected tissue becomes soft and rubbery and more prone to bending by external forces. In woody plants reduced lignification may also lead to a reduction in gravitropic response due to the involvement of lignification in the development of growth stresses. The effect of Cu deficiency on lignification occurs probably in the last step: the polymerisation of the lignin units. Two enzymes have been proposed for this step, a peroxidase (Boudet *et al.*, 1995) and laccase (Bao *et al.*, 1993; O'Malley *et al.*, 1993; Boudet *et al.*, 1995), though neither has been conclusively linked to lignin biosynthesis (Boudet *et al.*, 1995). Cu nutrition influences this part of lignin biosynthesis regardless of which enzyme is involved. Cu is present in diamine oxidase which provides the H_2O_2 for cell wall bound peroxidases linked to the biosynthesis of lignin (Marschner, 1995). Also the production of H_2O_2 necessary for peroxidase function is likely to involve CuZn superoxide dismutase (Turvey & Grant, 1990). CuZn superoxide dismutase is a ubiquitous enzyme occurring in the cytosol and mitochondria (Marschner, 1995). Its activity, the conversion of superoxide radicals

into peroxide and water, is necessary to all aerobic life forms. SOD prevents the oxidative damage caused by these highly reactive radicals which occur as by products of life processes.

Laccase, a polyphenol oxidase, was first isolated from fungi and occurs widely in higher plants. *Prunus persica* (Lehmann, *et al.*, 1974), the *Anacardiaceae* (Joel *et al.*, 1978), *Acer pseudoplatanus* (Bligny & Douce, 1983), *Pinus taeda* (Bao, *et al.*, 1993) and *N. tabacum* (Richardson & McDougall, 1996) have been shown to contain laccase. Cu has been reported as an integral part of the enzyme (Mayer, 1987). In *P. persica*, laccase contained 0.17% Cu equivalent to two atoms of Cu per molecule (Lehman *et al.*, 1974); four atoms Cu per molecule were reported in *A. pseudoplatanus* (Bligny & Douce, 1983). Cu concentration has been shown to affect the activity of laccase. The *in vitro* laccase activity of *A. pseudoplatanus* cell suspensions was roughly proportional to Cu concentration in the range of 2-100 µg/l suspension (Bligny *et al.*, 1986).

5.1.3.2 Hormonal control of gravitropic response

An auxin gradient across the leaning stem has been postulated as the cause of the gravitropic response (Went & Thimann, 1937). The Cholodny-Went Theory in its pure form has come under increased criticism as it cannot explain all responses to gravity and the involvement of other plant hormones of both a growth stimulating and inhibiting nature is generally accepted (Trewavas, 1992). Changes of the cytokinin concentration between upper and lower halves during gravitropic stimulation have been reported for roots and shoots of blackcurrant (El-Antably, 1975), and roots of corn and peanuts (Zhiyi *et al.*, 1989). An interaction between cytokinin and auxin is

indicated by the stimulation of zeatin ribose by applied NAA (Palni, *et al.*, 1988). Induced ethylene production has been linked with the gradient of auxin in the gravistimulated tissue and applied IAA increased ethylene production (Yu & Yang, 1979). A rapid increase of ethylene production has been reported during the gravitropic response of herbaceous plants (Lee *et al.*, 1990), while woody plants showed a slower and more sustained increase (Leopold *et al.*, 1972; Robitaille, 1975).

A reduction in the level of auxin in Zn deficient plants was first reported by Skoog (1940). The effects of Zn deficiency on auxin are numerous and include both inhibition of auxin synthesis and an increase in the oxidation of IAA (Brown *et al.*, 1993). Inefficient scavenging of superoxide radicals has been reported as one of the possible reasons for a reduction in IAA levels in Zn deficient bean plants (Marschner, 1995). Thus, Cu and Mn deficiency may also lead to a reduction in IAA levels, due to the inhibition of SOD activity. It is interesting to note that in the case of Mn both deficient and toxic levels of Mn lead to an increase in IAA oxidase activity in cotton (Morgan *et al.*, 1976), indicating that there are at least two ways in which Mn nutrition can affect IAA activity.

Cupric ions may be necessary for the promotion of a peroxidase system by cytokinins (Miller, 1985). This is an indication of a possible direct interaction between Cu and cytokinin activity. However, Cu may be more important in the regulation of cytokinin catabolism, as the *in vitro* activity of cytokinin oxidase, an enzyme catalysing the irreversible step in cytokinin catabolism, was stimulated by the addition of a Cu-complex (Chatfield & Armstrong, 1987). Cytokinin oxidase is thought to be a Cu

containing oxidase (Hare & van Stade, 1994). Low Cu nutrition may interfere with the synthesis of this enzyme.

5.1.4 Objectives of the experiment

The growth responses to the interaction between the application of Cu, Mn and Zn in Experiment 1 were not clear and may have been obscured by the effect of soil, N and P. In particular, the inhibiting effect of Mn on root growth and/or Cu uptake could not be established and the application rate may have been too low to elicit a response. Additional rates of nutrient application are used here to further investigate the interaction of Cu, Mn and Zn nutrition on growth and nutrient concentration of *E. nitens*. Larger pots allowed the seedlings to be grown for a longer time than in the previous experiments. The longer growing period may allow stem deformities to appear and to ascertain the effect of Cu, Mn and Zn application on them.

The experiment tested the following hypotheses:

- It is possible to induce the stem deformities in seedlings grown under accelerated conditions in the greenhouse.
- Application of Cu, Mn and Zn will affect the growth, stem form and nutrient composition of *E. nitens* seedlings with high N and P nutrition under controlled conditions.
- Application of Mn will affect root growth and Cu concentration of *E. nitens* seedlings.

5.2 Materials and Methods

5.2.1 Experimental design

A pot trial was undertaken in a greenhouse in 1996/97 and 1998/99. The trial had a 3³ factorial design with the following factors: three levels of added Cu (0 mg/kg, 8 mg/kg and 24 mg/kg), Mn (0 mg/kg, 10 mg/kg and 30 mg/kg) and Zn (0 mg/kg, 4 mg/kg and 12 mg/kg). The pots were arranged in a randomised complete block design with two blocks.

5.2.2 Soil and plant material

The soil in this experiment was the same as Soil 1 in Experiment 1 in Chapter 4. The soil was taken from the topsoil (0-10 cm) of a fertiliser trial where stem deformity had occurred due to suspected fertiliser-induced Cu deficiency (Turnbull *et al.*, 1994). Soil was sampled at three locations in the trial from plots where groups of both deformed and straight trees occurred. Soil was taken from the middle of groups of deformed trees. The soil was an organic, loamy sand. The soil was sieved through a 5 mm sieve, mixed well and air dried at 30^o C. The plant material used was seedlings of *E. nitens* from the same seed lot as in Experiment 1 of Chapter 4.

5.2.3 Preparation of pots

13.25 kg of soil were weighed into plastic bags. After weighing, the soil was mixed with 2 L deionised water. All pots received 240 mg/kg N and 160 mg/kg P. The nutrient salts for each treatment combination (Table 5.1) were mixed with acid washed sand (<0.5 mm) and ground finely in a ceramic mortar. Additional sand was ground in the mortar to clean it between treatments (altogether 140 mL of sand were used for

each pot). The nutrients and the sand were spread over the moistened soil and the soil and the nutrient mixture were mixed thoroughly. The soil was filled into black, plastic pots of 30 cm diameter and compressed to give a dry bulk density of 1.0 g cm^{-3} . The bottom of the pots was covered with washed river gravel separated from the soil by acid washed fly screen. After planting, the surface of the pot was covered with 5 mm acid washed sand (diameter between 0.5 and 2 mm). Pots were checked daily, and watered when the sand layer was dry.

Table 5.1: Reagents used for the experiment and milligrams added per pot

Reagent	low	medium	high
NH_2CONH_2	4545		
$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	8626		
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0	417	1250
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0	407	1222
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0	233	699

5.2.4 Plant propagation and management

The seeds of *E.nitens* were osmotically primed for 5 days in 2 % KNO_3 solution, washed and transferred to a sand tray using a pasteur pipette. When most seedlings had expanded their first true leaves, they were transplanted into the pots. Fifteen seedlings were planted in each pot in five groups of three (Figure 5.1). Measurements of plant height were started when plants had expanded three leaf pairs. Seedlings were

thinned to the largest in each group of three at the start of measurements (Plate 4.2) and to the largest in the pot five weeks after the start of measurements.

5.2.5 Plant harvest

Plants were harvested 27 weeks after measurements started. The youngest fully expanded leaves from each branch were used for nutrient analysis. These were washed with acid detergent (Sonneveld & van Dijk, 1982), rinsed three times with deionised water, drained and dried at 60°C to constant weight. Branches and stem leaves were

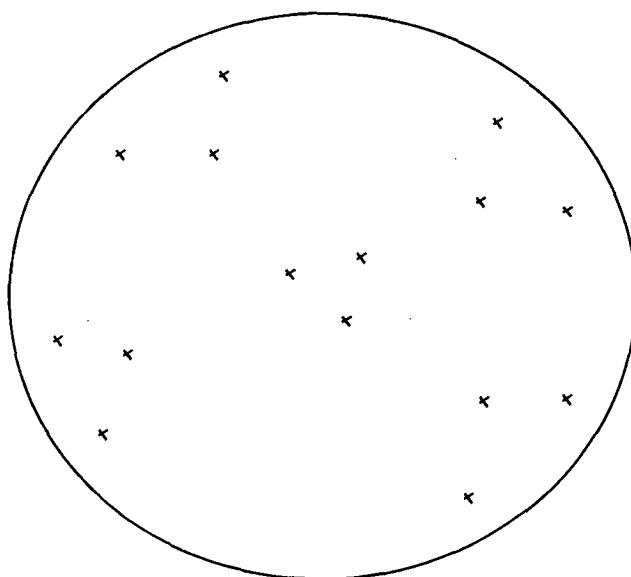


Figure 5.1: Arrangement of seedlings of *E. nitens* after transplanting to pots.

removed from the main stem and dried at 35°C. The leaves and branches were then separated by stripping the dry leaves from the branches. Branches and leaves were then dried at 60°C. The main stem was cut at the soil surface and dried at 60°C. Photographs were taken of trees prior to harvest and of stems after the branches had been removed. The roots were harvested as described in Chapter 4

5.2.6 Nutrient analysis

The plant and soil material was analysed for Cu, Mn and Zn concentration as described in Chapter 4.

5.2.7 Statistical analysis

The data was analysed by Analysis of Variance using Genstat 5. The statistical model used was a fixed effect model (Model I) (Steel & Torrie, 1980). There were no significant interactions between treatments and only the main effects are presented.

5.3 Results

5.3.1 Effect of Cu, Mn and Zn application on the growth of *E. nitens* seedlings

There was a significant increase of height due to Cu application starting 9 weeks after measurements commenced (Figure 5.2). Cu application resulted in a significant increase in the dry mass of all plant parts at harvest (Table 5.2). The increase was particularly strong for leaves and fine roots. For these plant parts the dry mass increased up to 40 % due to the application of Cu. The increase in the dry mass of stems, branches and coarse roots with applied Cu was less than 25%. There was no significant difference in height and dry mass between 8 and 24 mg/kg applied Cu. Application of Mn resulted in an increase in the dry mass of roots. This effect was significant for fine roots and the total root dry mass. The effect of applied Mn on fine roots was stronger than that of applied Cu and the dry mass increased by 44 % from 16.5 g with no Mn added to 23.8 g with 30 mg/kg Mn added. There was no significant effect of Zn application on dry mass and height.

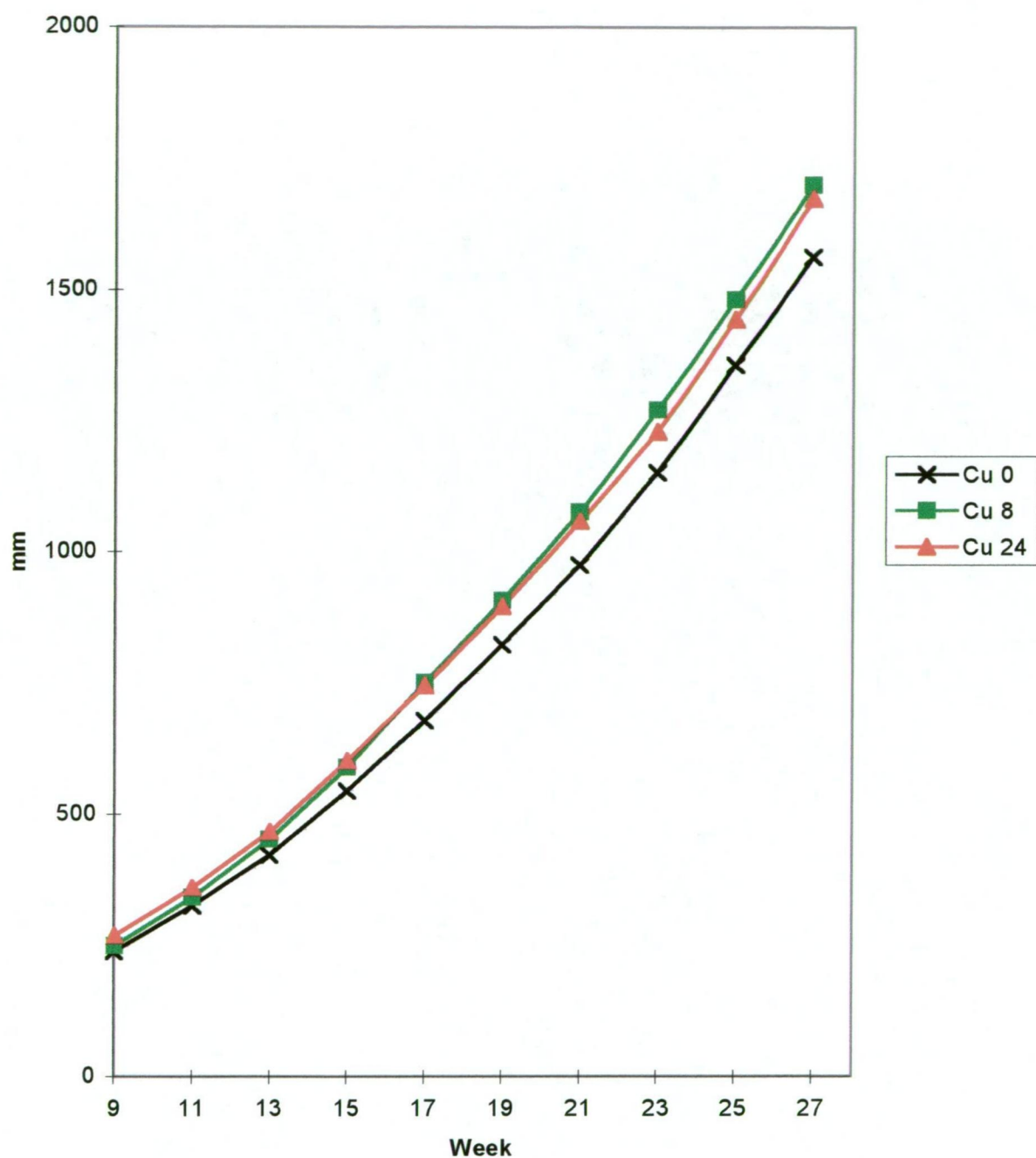


Figure 5.2: The effect of Cu application on the height of *E. nitens* seedlings measured at fortnightly intervals. Weeks were counted from the start of the measurements. From week 9 onwards a significant increase in height due to the application of Cu was detected. There were no significant differences in height between 8 or 24 mg/kg applied Cu. (n=18)

Table 5.2: Main effects of Cu, Mn and Zn application on the dry mass (in g) of *E. nitens* seedlings. Bold numbers indicate significantly greater values (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, $n=18$)

	Cu added (mg/kg)			Mn added (mg/kg)			Zn added (mg/kg)		
Dry mass (g)	0	8	24	0	10	30	0	4	12
Fine roots (< 1mm)	16.7	23.6***	22.2**	16.5	22.3**	23.8**	20.8	21.6	20.2
Coarse roots (> 1mm)	22.3	27.8**	27.3**	24.2	26.7	26.5	26.5	27.2	23.8
Branches	58.4	69.0***	71.9***	64.4	68.3	66.6	68.0	66.6	64.8
Main stem	78.9	98.2***	93.6***	93.1	91.1	86.4	86.2	89.8	94.7
Leaves	137	175***	191***	159	170	174	167	166	169
Whole plant	314	393***	406***	357	378	377	369	371	373
Shoots	275	342***	356***	317	329	327	321	323	329
Roots	39.0	51.5***	49.5***	40.7	49.0*	50.3*	47.3	48.8	44.0

5.3.2 Effects of Cu, Mn and Zn application on Cu, Mn and Zn concentration in seedlings of *E. nitens* and soil

Cu, Mn and Zn application increased the concentration of the respective nutrients in roots and leaves of *E. nitens* seedlings (Table 5.3). The strongest effect was that of Cu on the Cu concentration of leaves which increased more than eightfold from 0.6 to 5.0 mg/kg with the application of 24 mg/kg Cu. Zn concentration of leaves was least affected and only at the high (12 mg/kg) rate of Zn application was there a significant increase from 21.9 to 26.0 mg/kg. Cu application resulted in a reduction of Mn concentration in roots and leaves. The reduction of leaf Mn concentration was stronger and leaves with applied Cu had only approximately two-thirds of the foliar Mn concentration of plants without applied Cu.

The root : soil ratio of Cu and Mn concentration was reduced by the application of the respective nutrient (Table 5.4). Cu application reduced the ratio significantly from 1.93 without applied Cu to 1.17 with 10 mg/kg and to 0.91 with 24 mg/kg applied Cu. Only the high Mn application rate (30 mg/kg) resulted in a significant reduction of the ratio from 13.01 to 9.73. Cu application reduced the root : leaf ratio of Cu concentration significantly from 9.17 to 4.79 and 4.13 for 8 and 24 mg/kg applied Cu respectively. There was no significant difference in ratio between 8 and 24 mg/kg applied Cu. Cu application increased the root : leaf ratio of Mn concentration significantly. The root : leaf ratio of Zn concentration more than doubled from 7.51 to 19.33 with the application of 12 mg/kg Zn.

Table 5.3: Main effects of Cu, Mn and Zn application on the Cu, Mn and Zn concentration (in mg/kg) in roots and leaves of *E. nitens* seedlings. Different letters for each nutrient indicate significantly ($p < 0.001$) different values. ($n = 18$)

Nutrient concentration (mg/kg)		Cu added (mg/kg)			Mn added (mg/kg)			Zn added (mg/kg)		
		0	8	24	0	10	30	0	4	12
Cu	Roots	5.1 ^a	10.8 ^b	20.0 ^c	12.7	11.2	12.0	12.1	11.7	12.2
	Leaves	0.6 ^a	2.4 ^b	5.0 ^c	2.9	2.4	2.7	2.6	2.7	2.7
Mn	Roots	272	247	245	179 ^a	248 ^b	336 ^c	258	252	254
	Leaves	1233 ^b	809 ^a	876 ^a	665 ^a	939 ^b	1314 ^c	1004	991	922
Zn	Roots	299	283	293	304	286	285	157 ^a	232 ^b	486 ^c
	Leaves	24.4	23.2	24.4	25.7	22.8	23.5	21.9	24.1	26.0

Table 5.4: Main effect of Cu, Mn and Zn application on the ratios of Cu, Mn and Zn concentration between soil, roots and leaves of *E. nitens* seedlings. Different letters for each nutrient indicate significantly ($p < 0.05$) different values.

	Ratios between nutrient concentrations	Cu added (mg/kg)			Mn added (mg/kg)			Zn added (mg/kg)		
		0	8	24	0	10	30	0	4	12
Cu	Root : soil	1.93 ^c	1.17 ^b	0.91 ^a	1.46	1.27	1.28	1.37	1.33	1.32
	Root : leaf	9.17 ^b	4.79 ^a	4.13 ^a	5.82	6.30	5.96	5.98	5.82	6.29
Mn	Root : soil	11.62	10.88	12.28	13.01 ^b	12.04 ^b	9.73 ^a	11.93	11.57	11.28
	Root : leaf	0.23 ^a	0.32 ^b	0.29 ^{a,b}	0.29	0.28	0.27	0.27	0.27	0.30
Zn	Root : soil	11.73	9.52	9.49	11.16	10.10	9.48	10.16	9.18	11.41
	Root : leaf	12.65	12.36	11.93	12.10	12.52	12.32	7.51 ^a	10.09 ^a	19.33 ^b

A depletion zone of Cu concentration developed near the roots with increasing Cu application (Table 5.5). The depletion was significant when 24 mg/kg Cu was applied and rhizosphere soil had a 2.1 mg/kg lower Cu concentration than the bulk soil. Mn and Zn concentration showed the opposite trend and accumulated in the root zone. There was no significant effect of nutrient application on the accumulation of Mn in the root zone. Each increase in Zn application resulted in a significantly higher accumulation of Zn in the root zone (11.8, 21.0 and 30.2 mg/kg for 0, 4 and 12 mg/kg applied Zn respectively). The accumulation of Zn in the rhizosphere was also increased by Cu application. The difference was only significant between no applied Cu (16.6 mg/kg) and 24 mg/kg applied Cu (24.1 mg/kg).

5.3.3 Effect of Cu, Mn and Zn application on the stem form of *E. nitens* seedlings.

While most seedlings showed a straight growth habit and displayed little lean, there was a large degree of variation in stem form between the trees and a small number of seedlings had considerable lean and some had strong kinks or double leaders. No significant differences in the lean of stems or the number and severity of stem deformities could be related to nutrient applications. There was no relationship of the stem deformities to the amount of micronutrients applied and duplicate trees receiving the same nutrient combinations did not show similar degrees of deformity. None of the symptoms were as severe as some encountered in the field.

Table 5.5: Main effect of Cu, Mn and Zn application on the Cu, Mn and Zn concentration (in mg/kg) in bulk soil and rhizosphere and the accumulation of the nutrients in the rhizosphere. Different letters for each nutrient indicate significantly different values. The significance was $p < 0.05$ for the effect of Cu application on Cu and Zn accumulation and Zn rhizosphere concentration. All other values were different to $p < 0.001$ (n=18)

		Cu added (mg/kg)			Mn added (mg/kg)			Zn added (mg/kg)		
		0	8	24	0	10	30	0	4	12
Cu	Bulk soil	2.4 ^a	9.8 ^b	24.3 ^c	12.0	12.2	12.3	12.1	12.0	12.4
	Rhizosphere	2.7 ^a	9.3 ^b	22.2 ^c	10.7	11.6	11.9	11.3	11.2	11.7
	Accumulation	0.2 ^b	-0.5 ^{a,b}	-2.1 ^a	-1.38	-0.6	-0.4	-0.8	-0.8	-0.7
Mn	Bulk soil	18.9	17.5	19.0	10.8 ^a	16.6 ^b	28.3 ^c	18.1	18.6	18.7
	Rhizosphere	23.8	23.4	24.0	14.3 ^a	21.7 ^b	35.2 ^c	23.3	23.9	24.0
	Accumulation	4.9	5.9	5.0	3.5	5.4	6.9	5.1	5.4	5.3
Zn	Bulk soil	9.4	9.2	9.5	9.3	9.4	9.3	5.3 ^a	8.4 ^b	14.3 ^c
	Rhizosphere	26.0 ^a	31.4 ^{a,b}	33.6 ^b	28.6	30.8	31.6	17.0 ^a	29.5 ^b	44.5 ^c
	Accumulation	16.6 ^a	22.2 ^{a,b}	24.1 ^b	19.3	21.3	22.3	11.8 ^a	21.0 ^b	30.2 ^c

5.4 Discussion

5.4.1 Seedling growth

The stimulating effect of Cu application on all aspects of seedling growth in this experiment and the absence of such a consistent response for the other nutrients reinforces the results from Chapter 4, where Cu was indicated as the primary micronutrient affecting the growth of *E. nitens* seedlings. All seedlings without applied Cu had Cu concentrations below 1 mg/kg, significantly lower than the concentration of 1.4 mg/kg considered critical by Turnbull *et al.* (1994). The low rate of Cu application (8 mg/kg) increased the foliar Cu concentration to a level above 1.4 mg/kg. The increase in seedling growth when 8 mg/kg Cu was applied supports the conclusion of Turnbull *et al.* (1994) that a foliar concentration of Cu below 1.4 mg/kg can be considered deficient in *E. nitens*. The absence of a growth response to the higher Cu application rate indicates that there was a luxury supply of Cu at this rate (24 mg/kg). There was no growth response to Zn application when Cu supply became non-limiting, indicating that Zn deficiency is not likely to be a factor affecting the growth of young seedlings in this experiment.

There was an increase of fine roots with application of Mn, although the root Mn concentration was double that of the roots affected by lesions in the field (Chapter 3). A possible conclusion is that Mn toxicity is not the cause of root lesions and stunted root systems observed in the field. On the other hand the Mn toxicity may have been localised only in the part of the plantation where root systems were excavated for the research in Chapter 3. None of the roots found during the soil collection for the greenhouse experiments showed the lesions.

5.4.2 Cu, Mn and Zn concentrations in seedlings and soil

The nutrient concentrations of Cu and Mn in both roots and leaves responded strongly to the application of the respective nutrients, while only the Zn concentration of roots increased significantly with Zn application and Zn concentration in leaves showed only a statistically non significant increase even at the high rate of Zn application. This rate was higher than that used in Experiment 1 of Chapter 3. It is interesting to note that the leaf concentrations are lower than those in Experiment 1 of Chapter 3, while the root concentrations are higher. This indicates that some of the Zn accumulated by the roots cannot be transported into the shoot and may be present as insoluble compounds, for example phosphates.

There was a reduction in root : soil ratio of Cu and Mn with increasing application of the respective nutrient. Without applied Cu, an accumulation of Cu in the roots prior to the transport into the shoot was evidenced by the high root : leaf ratio in Cu concentration without applied Cu. A result of this accumulation was a high root : soil ratio without applied Cu. This indicates that the mechanism of the fertiliser-induced Cu deficiency occurs more likely during the root to shoot transport within the plant than during the uptake into the root. At a high soil Cu concentration the ratio was close to 1 and did not change much when the higher rate of Cu was applied. This suggests that at low Cu concentration an active uptake mechanism is present that helps maintain the higher relative concentration in the roots. At the higher Cu soil concentration passive uptake may be more important.

With applied Cu, the Cu concentration in the soil near the roots was lower than that in the bulk soil indicating the development of a depletion zone (Barber, 1995). This depletion zone is an indication that the applied Cu has been bound by soil solids and that transport in the mass flow of soil solution is lower than the uptake into the root. At the highest application rate, the soil near the root surface had a significantly lower Cu concentration than the bulk soil. This shows that although higher uptake occurred due to a high Cu concentration at the root surface, the concentration of the soil solution in the bulk soil was not sufficiently raised to maintain the concentration by mass flow. Cu that was not readily available in the bulk soil was released near the root surface indicating a lower soil solution concentration. The Cu that was released in the rhizosphere soil was likely a portion of the applied Cu. The slower release of Cu from the bulk soil and the increased supply of Cu from the soil near the root surface is one of the reasons that Cu fertilisation has a strong residual effect (Shuman, 1998).

Zn accumulated in the root zone. Thus Zn was not bound strongly by soil solids and at the highest Zn application rate, supply by mass flow exceeded uptake. The difference in the response to applied Cu and Zn occurs because Cu binds preferentially to soil organic matter, while Zn is bound by inorganic soil colloids (McLaren *et al.*, 1981; Barak & Helmke, 1993). The soil in this experiment had a high organic matter content and contained only little clay. The increased accumulation of Zn due with increased level of Cu application can be explained by two mechanisms. Firstly, the increase in leaf biomass with Cu application may lead to an increase in transpiration and thus an increase in mass flow of Zn. Secondly, an increase in soil Cu may lead to competition between Cu and Zn for binding sites and reduced retention of Zn in the soil.

5.4.3 Stem form of seedlings

It was not possible to replicate the stem deformities that were observed in *E. nitens* in the field during this greenhouse experiment. Limitations of the experiment, which could have resulted in an insufficient induction of Cu deficiency may partly explain the absence of differences in stem form due to micronutrient applications. N and P were applied at a rate equivalent to the lowest at which stem deformities can be expected in the field (300 kg/ha N and 150 kg/ha P, C. Turnbull, pers. comm.). This rate was chosen to make it more likely that induced deformities could be ameliorated by application of Cu and Zn, but it may have been insufficient to induce sufficient Cu deficiency. Another limiting factor was the time that it was possible to maintain growth in the pots and the continuous growth rate. In the field deformities occurred at the start of the second growing season (Turnbull *et al.*, 1994). This may indicate that deformities are the result of an insufficient reserve in the trees to supply the high demand of Cu in the rapid spring growth of the second year.

Neither of these explanations is very satisfactory as very low foliar (< 1 mg/kg) Cu concentrations were measured in the plants without applied Cu. These foliar concentrations were lower than those of many trees showing stem deformities in the field (Turnbull *et al.*, 1994). It is therefore unlikely that insufficient Cu deficiency was the reason that the seedlings in the greenhouse did not show stem deformities. It is more likely that stem deformities failed to occur in the greenhouse because of the absence of environmental factors such as wind in the controlled greenhouse environment. Environmental factors have been associated with the occurrence of stem deformities in *P. radiata* (Jacobs, 1939; Turvey *et al.* 1992). It is therefore possible that Cu deficiency only predisposes trees to the effects of these environmental factors

and is by itself not a sufficient cause of the deformity. Another important aspect is the age of the trees. In the field, deformities occurred at the start of the second growing season and the seedlings in the greenhouse were only eight months old (from germination). As the tree grows the loads increase faster than the load bearing stem. This may mean that trees have to reach a certain critical size before stem deformities occur. Also there was a high degree of variability in the stem form of seedlings, which may have obscured the effect of nutrition. A strong genetic influence on the expression of stem deformity has been previously found for *P. radiata* (Pederick *et al.*, 1984; Bail & Pederick, 1989).

Chapter 6: Effect of N and Cu nutrition on the distribution of Cu in *E. nitens* clones

6.1 Introduction

6.1.1 Background

Apart from a reduction in the total uptake of micronutrients into the plant, high fertiliser application may also affect the distribution of micronutrients in the plant. If the micronutrient is immobilised in one tissue it may not be available to some growing tissues when the nutrient supply becomes limiting. Cu mobility in plants has been closely linked to N nutrition (Bergmann, 1992). In particular the remobilisation of Cu from senescent leaves and the rate of senescence have been shown to be influenced by the amount of N supplied (Hill *et al.*, 1978). A reduction of senescence and remobilisation due to high N application could be a possible mechanisms by which high N nutrition leads to a temporary Cu deficiency during the high demand at the start of the second growing season of *E. nitens*.

6.1.2 Cu distribution in roots and shoots

The results from a number of studies reporting concentrations and content of Cu in the roots and shoots of plants have been summarised in Table 6.1. The concentration in the roots is usually two to three times higher than in shoots, but in some species the difference can be even higher. Smilde (1973) reported Cu concentrations in roots of *Salix alba* that were 10 – 20 times higher than the shoot concentration. As an exception, Chaudhry & Loneragan (1970) found similar Cu concentrations in shoots and roots of *T. aestivum* without applied Cu, but when Cu was applied the root

Table 6.1 Cu concentration (in mg/kg) and Cu content (μg per plant) of a number of plant species. The ranges of concentrations and contents are the lowest and highest in the respective experiments. In experiments with several harvests dates, only the harvest with the oldest plants was considered. (* denotes contents that had not been reported and were calculated from yield and concentration)

Species	Plant age	Cu applied (mg/kg)	Growth medium	Concentration (mg/kg)		Content (μg per plant)		Reference
				Root	Shoot	Root	Shoot	
<i>Lolium perenne</i>	21 days	0 – 10	Solution	7.7 - 3263	3.8 - 24.6	43.9 – 10115	67.8 – 419.5	Jarvis (1978)
<i>L. perenne</i>	42+ days	0	Soil	14 - 42	4.7 – 6.8	33 – 176	13 - 94	Jarvis & Whitehead (1981)
<i>Pinus radiata</i>	6 months	?	Sand	7.3 - 17.8	3.3 - 5.7	3.8 – 4.2	4.2 – 8.2	Olykan & Adams (1995)
<i>Pinus silvestris</i>	23 months	1.8	Soil	27 - 48	4.8 - 13.6	113 – 1648*	41 – 645*	Smilde (1973)
<i>Populus euramericana</i>	6 months	1.8	Soil	13 – 42	3.5 - 14.9	72 – 419*	83 – 406*	Smilde (1973)
<i>Pseudotsuga menziesii</i>	15 months	1.8	Soil	23 - 38	7.2 - 12.2	135 – 252*	110 – 371*	Smilde (1973)
<i>Salix alba</i>	3 months	1.8	Soil	58 - 484	6 - 26	46 – 2806*	38 – 672*	Smilde (1973)
<i>Trifolium pratense</i>	70 days	0 – 0.16	Solution	30.5	10.0	0.469	0.417	Hill (1973)
<i>Triticum aestivum</i>	66 days	0 - 1.8	Soil	1.2 – 9.5	0.9 – 3.2	0.82 – 4.03	0.55 – 5.09	Chaudhry & Loneragan (1970)
<i>T. aestivum</i>	21 days	0.02 - 0.06	Solution	56 – 136	20 - 28	19.8 – 47.6*	23.1 – 37.2*	Racz & Haluschak (1974)

concentration was again higher. Other studies of the same species (Racz & Haluschak, 1974; Jarvis & Robson, 1982) reported Cu concentrations in the roots that were 2-3 times higher than those in the shoots at a low level of Cu application.

The distribution of Cu between root and shoot differs with plant age and Cu nutrition . Jarvis and Robson (1982) reported higher concentration in roots vs. shoots of 26-day old *Trifolium pratense* plants grown in solution culture. At age 60 days however, Cu concentration in roots and shoots was similar. Jarvis (1978) reported that the concentration in roots of *Lolium perenne* grown in nutrient solution containing no added Cu had double the Cu concentration of the shoots. When plants were grown in nutrient solution containing 10 mg/kg added Cu, the concentration in roots was more than a hundred times higher than in shoots. The proportion of Cu contained in the roots increased from 39% without added Cu to 96% with 10 mg/kg Cu added. Retention of excessive levels of Cu in the roots of plants is one of the mechanisms by which plants can tolerate high metal levels (Farago, 1981).

Generally the biomass of shoots is greater than that of roots. Therefore the picture is not as clear cut when the Cu content of roots and shoots is considered. Similar Cu contents for roots and shoots were shown for *Populus euramericana* (Smilde, 1973), *Pseudotsuga menziesii* (Smilde, 1973), *T. pratense* (Hill, 1973) and *T. aestivum* (Chaudhry & Loneragan, 1970; Racz & Haluschak, 1974). *P. radiata* had almost double the shoot Cu content compared to the root content at the high end of the range (Olykan & Adams, 1995). The other species shown in Table 6.1 have a higher content of Cu in the roots than in the shoots. The differences between the species depend on two factors: firstly on how strong the difference between the Cu concentration in the

roots and the shoots was and on the inherent shoot: root ratio of the species. Generally, it appears that close to half and often more than half of the Cu taken up by the plant is contained in the roots. Factors affecting the retention of Cu in the roots may therefore play an important role in induced Cu deficiency.

Within roots, Cu was mostly associated with the cell walls (Jarvis, 1978). Of the above-ground parts, most Cu is usually contained in the leaves (Gupta, 1990), where it is found in Fraction I protein (Rubisco) and in plastocyanin as part of the photosynthetic electron transfer chain (Loneragan, 1981).

6.1.3 Mobility of Cu in plants

Cu has been described as variably mobile within the plant and this mobility as being dependent on the Cu and N nutrition of the plant. Cu was found to be mobile in plants with adequate Cu nutrition and immobile in deficient plants (Loneragan, 1981). Cu deficiency symptoms generally occur first in young plant parts (Bussler, 1981), an indication of restricted mobility from old to new leaves, but significant Cu concentrations may still be measured in phloem sap (Loneragan, 1981). Bukovacz and Wittwer (1957) found Cu to be of intermediate mobility when they applied radioisotopes of 14 elements to the leaves of *P. vulgaris* plants and measured the distribution of radioactivity 24 hours after the application.

6.1.4 Cu transport in xylem and phloem

Cu is present in both xylem and phloem sap (Mullins *et al.*, 1986). In both cases it is present mainly as a complex with a number of ligands. Computer programs originally developed for the speciation of metal cations in soil solution have been used to model the speciation of metal ions in xylem and phloem sap (Mullins *et al.*, 1986; White *et al.*, 1981). These programs use databases containing established stability constants to calculate equilibrium concentrations which will satisfy all known metal/ligand combinations. The reliability of these models depends on the accuracy of the database used and the number of complexes considered (White *et al.*, 1981). Nicotiane amine (NA) was postulated as a specific transport molecule responsible for Cu transport in *L. esculentum* xylem (Pich & Scholz, 1996) and *R. communis* phloem (Schmidke & Stephan, 1995). These studies used a NA deficient mutant of *L. esculentum* or removed endogenous NA by decapitating *R. communis* seedlings and were able to demonstrate inhibition of Cu transport.

6.1.5 N application and Cu concentrations in plants

The effect of N nutrition on Cu concentrations in plants is variable and depends on the level of Cu nutrition. An optimal N nutrition promoted the uptake of Cu in *P. gratissima* leaves (Lahav *et al.*, 1990), but high nutrition of N in *T. aestivum* on soil with marginal Cu supply induced Cu deficiency (Brennan, 1993). The response of forest trees to N and Cu application differed between species studied (van den Burg, 1983), but the general trend was that an increase of foliar Cu concentration with higher N application depended on the simultaneous application of Cu.

6.1.6 Effect of leaf senescence on the retranslocation of Cu in plants

The effect of leaf senescence on the distribution and remobilisation of Cu has been studied comprehensively in *T. aestivum* (Hill *et al.*, 1978, 1979a,b; Loneragan *et al.*, 1980). It was shown that the translocation of Cu from older to younger leaves and the grain depended on the onset of senescence. Treatments delaying senescence, like Cu deficiency (Hill *et al.*, 1979a) and luxury supply of N (Hill *et al.*, 1978), reduced the remobilisation of Cu. Induction of senescence, for example by shading of the oldest leaf (Hill *et al.*, 1979b) or low N supply, resulted in high mobility of Cu. Shading of oldest leaves resulted in a transient increase of the Cu concentrations of younger leaves (Hill *et al.*, 1979b). All these results indicate that the effect of N application on the senescence and remobilisation of Cu are likely to lead to N-induced Cu deficiency in the growing tissues. As there was evidence in Chapter 4 (Plate 4.3) that this occurs in *E. nitens* this may be a possible mechanism of the fertiliser-induced Cu deficiency.

6.1.7 Objectives of the experiment

The experiment described in this chapter studied the effect of N and Cu nutrition on the distribution of Cu in eucalypt clones. Two questions were of particular interest considering the previous results from other plant species. What is the effect of N and Cu nutrition on the retention of Cu in the roots and its distribution between roots and shoots and its supply to growing shoots? Secondly, what is the effect of Cu and N nutrition on the rate of senescence of old leaves and the translocation of Cu to young growing leaves? Visual observations from a previous experiment indicated that low levels of N resulted in increased leaf senescence (Plate 4.3).

The experiment tested the following hypotheses:

- Low concentrations of Cu in the soil lead to retention of Cu in the roots.
- Cu deficiency and high N nutrition lead to a reduction in leaf senescence and retention of Cu in the leaf during senescence resulting in reduced remobilisation of Cu within the plants.
- Different clones will show differing behaviour in Cu uptake and distribution.

6.2 Materials and methods

6.2.1 Experimental design

A pot trial was undertaken in a greenhouse between October 1998 and February 1999. The trial had a 2x2x4 factorial design with the following factors: 2 clones of *E. nitens*, 2 levels of added N (24 mg/kg, 240 mg/kg), 4 levels of added Cu (0 mg/kg, 4 mg/kg, 10 mg/kg, 25 mg/kg). The pots were arranged in a randomised complete block design with 4 blocks.

6.2.2 Soil and plant material

The soil for the experiment was taken from the topsoil (0-10 cm) of a fertiliser trial where stem deformity occurring due to fertiliser-induced Cu deficiency has been reported (Turnbull *et al.*, 1994). At three locations in the trial, soil was sampled in the middle of groups of trees showing deformity. The soil was an organic, loamy sand with the following properties: pH (1:5 water) 3.8; LOI 8 %; total Cu (HNO₃/H₂O₂ digest) 2 mg/kg. The soil was sieved through a 5 mm sieve, mixed well and air dried at 30^o C. The plant material used was two *E. nitens* clones (E 533 and E 534) obtained from North Forest Products Ltd (Burnie, Tasmania). One plant was grown per pot. At

the start of the experiment, plants were pruned to retain a similar number of leaves. The number of branches was kept equal in each block.

6.2.3 Preparation of pots

3 kg of air-dried soil for each pot were weighed out and moistened to reduce the water repellence of the dry soil. The nutrient salts for each treatment combination plus 160 mg/kg P for each pot were mixed with acid washed sand (<0.5 mm) and ground finely in a ceramic mortar. Additional sand was ground in the mortar to clean it between treatments (altogether 140 mL of sand were used). The nutrients and the sand were spread over the moistened soil and mixed well. The soil was filled into black, plastic pots with 20 cm diameter and compressed to 3.0 L to give a dry bulk density of 1.0 g cm^{-3} . After planting of the clones, the surface of the pot was covered with 1 cm acid washed sand (diameter between 0.5 and 2 mm). Pots were checked daily and watered when the sand layer was dry.

6.2.4 Plant harvest

Senescing leaves were collected throughout the experiment. This was done when it was possible to remove them with gentle manipulation. The material was air dried and stored in paper bags in the greenhouse. Whole plants were harvested when enough senescent leaves for nutrient analysis had been collected from all treatments. The expanding tips up to the first fully expanded leaves were harvested for nutrient analysis. Tips that were not expanding or showed any damage were not used. Tips and senescent leaves were washed with acid detergent (Sonneveld & van Dijk, 1982), rinsed three times with deionised water, drained and dried at 60°C to constant weight. The rest of the plants were harvested for biomass determination. Shoots were cut at

the surface of the soil and the whole plants were dried at 60 °C. After they were dried, plants were divided into stems and leaves, dried again at 60 °C and weighed.

Roots were harvested as described in Chapter 4

6.2.5 Nutrient analysis

The soil and plant materials were analysed for Cu, Mn and Zn concentrations as described in Chapter 4

6.2.6 Calculation of Cu content

The Cu concentration in the tips and fine roots were multiplied by the dry mass of the shoots and roots, respectively, to estimate the Cu content of the tissues. Calculation of the content by this method is likely to overestimate the Cu content in the plants, because both the tips and the fine roots can be expected to have higher Cu concentrations than the other tissues of shoots (as reported for vegetables in Huett *et al.*, 1997) and roots (see Table 3.8 on page 64 of this thesis) respectively. However, the relative amounts will be less affected as both plant parts are overestimated. This study is mainly interested in investigating the relative amounts between the two plant parts and the estimate calculated by this method should give sufficiently accurate results.

The remobilised Cu was estimated by multiplying the difference in concentration of tips and senescent leaves by the dry mass of senescent leaves. This method is based on the assumptions that firstly there is no difference in the Cu concentration of the growing shoot tip and the mature leaf and, secondly, that there is no loss of dry mass

during senescence. The calculated amount is therefore only a rough estimate as the Cu concentration is likely to have changed throughout the life of the plant and leaf dry mass would be lost during senescence. Nonetheless, the estimate will be an indication of how important remobilisation is for the Cu nutrition of *E. nitens* seedlings and if there is a strong effect of N application.

6.2.7 Statistical analysis

The data was analysed by Analysis of Variance using Genstat 5. The statistical model used was a fixed effect model (Model I) (Steel & Torrie, 1980).

6.3 Results

6.3.1 Biomass.

6.3.1.1 Main effects.

Harvested dry mass of the two clones differed (Table 6.2). Clone E 533 had significantly greater dry mass of total roots, while E 534 had significantly greater dry mass of fine roots. The dry mass of all plant parts shown was significantly higher at high N .

Table 6.2: Main effects of clone and N application on the dry mass (in g /plant) of some plant parts. Only main effects that were not affected by interactions are shown. Bold numbers are significantly higher. (* = p<0.05, ** = p<0.01, *** = p<0.001). n =

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	Clone			N added (mg/kg)		
	E533	E 534		24	240	
Fine roots	3.8	4.3	**	3.6	4.6	***
Total roots	11.4	10.5	*			
Stems	22.9	21.9		20.1	24.8	***
Total shoots	65.6	68.9		58.4	76.1	***
Total plant	77.0	79.3		69.7	86.6	***

6.3.1.2 Effects of interactions

6.3.1.2.1 Clone x N

There was a significant interaction between clone and N application on the dry mass of leaves. There was no significant difference in leaf dry mass between the clones at low N (Table 6.3). At high N, leaf dry mass increased significantly from 33.5 to 41.1 g for E 533 and from 34.7 to 51.9 g for E 534. This resulted in a significantly higher dry mass of leaves for E 534 with high N.

6.3.1.2.2 Clone x Cu

There was a significant interaction between clone and Cu application on the dry mass of coarse roots. There was no significant difference in the dry mass of coarse roots for E 533 due to Cu application (Table 6.4). For E 534 dry mass of coarse roots was reduced with increasing Cu application. E 534 with 10 and 25 mg/kg applied Cu had significantly lower coarse root dry mass than E 534 without applied Cu. At 10 and 25 mg/kg applied Cu, E 534 had significantly lower coarse root dry mass than E 533.

Table 6.3: The effect of the interaction between clone and N application on the dry mass (g), Cu concentration (mg/kg) and the Cu content (in µg/plant) of different plant parts. The Cu content of shoots and whole plants was estimated by using concentrations measured in the shoot tips (up to first fully expanded leaf) and fine roots. Remobilised Cu was estimated by multiplying the difference of the concentration of tips and senescent leaves with the dry weight of the senescent leaves. Different letters within each row indicate significant differences. (n = 16)

	Clone	E 533		E534		l.s.d.
	N added (mg/kg)	24	240	24	240	(p<0.05)
Dry weight (g/plant)	Roots	11.9 ^b	10.9 ^{a, b}	10.8 ^a	10.1 ^a	1.0
	Leaves	33.5 ^a	41.1 ^b	34.7 ^a	51.9 ^c	4.7
	Shoots	58.8 ^a	72.5 ^b	58.0 ^a	79.7 ^c	6.0
	Total	70.7 ^a	83.4 ^b	68.8 ^a	89.8 ^b	6.7
Cu concentration (mg/kg)	Fine roots	13.5 ^b	11.8 ^a	11.6 ^a	11.4 ^a	1.0
	Tips	4.3 ^a	4.8 ^b	5.5 ^c	6.7 ^d	0.4
	Senescent leaves	2.2 ^a	2.8 ^b	2.1 ^a	2.7 ^b	0.3
Cu content (µg/plant)	Roots	162 ^c	131 ^b	126 ^{a, b}	105 ^a	22
	Shoots	252 ^a	364 ^b	321 ^b	539 ^c	59
	Senescent leaves	13.2	18.1	13.1	15.4	6.1
	Whole plants	427 ^a	513 ^b	460 ^{a, b}	660 ^c	68
	Remobilised Cu	12.4 ^a	11.2 ^a	21.2 ^b	22.8 ^b	7.8

Table 6.4: The effect of the interaction between clone and Cu application on the dry mass (in g) of coarse roots and the Cu content of roots (in µg) Different letters indicate significant differences (n = 8).

		Cu0	Cu4	Cu10	Cu24
Coarse root dry mass (g)	E533	7.25 ^{b,c}	7.85 ^c	7.36 ^{b,c}	7.69 ^c
	E534	7.35 ^{b,c}	6.30 ^{a,b}	5.69 ^a	5.53 ^a
Root Cu content (µg)	E533	61.2 ^a	108.1 ^b	143.3 ^c	273.2 ^c
	E534	60.4 ^a	84.1 ^{a,b}	111.9 ^b	206.3 ^d

6.3.1.2.3 *N x Cu*

There was a significant interaction between N and Cu application on the dry mass of roots and leaves. Plants grown at high N had significantly lower coarse root mass than at low N with 4, 10 or 25 mg/kg applied Cu (Table 6.5). There were no other significant differences for coarse root mass due to N and Cu application. There was a significant decrease of total root mass at high N with the two highest levels of applied Cu and no significant difference between the other treatments. At low N, there was no significant effect of Cu application on the dry mass of leaves. At high N, there was a trend towards higher leaf mass with higher Cu application. When 10 and 25 mg/kg Cu were applied the difference was significant.

Table 6.5: The effect of the interaction between N and Cu application on the dry mass (in g/plant), Cu concentration (in mg/kg), and the ratio between Cu concentrations of plant parts. Different letters within each row indicate significant differences. (n = 8)

	N added (mg/kg)	24				240			
	Cu added (mg/kg)	0	4	10	25	0	4	10	25
Dry weight (g/plant)	Coarse roots	7.3 ^b	8.3 ^b	7.9 ^b	7.9 ^b	7.3 ^b	5.9 ^a	5.2 ^a	5.3 ^a
	Roots	10.8 ^{a, b}	11.8 ^b	11.2 ^b	11.5 ^b	11.8 ^b	10.7 ^{a, b}	9.7 ^a	9.9 ^a
	Leaves	34.7 ^a	34.2 ^a	34.0 ^a	33.3 ^a	39.6 ^{a, b}	43.0 ^b	50.0 ^c	53.4 ^c
Cu concentration (mg/kg)	Fine roots	6.5 ^b	9.3 ^c	12.9 ^e	21.6 ^f	4.4 ^a	7.6 ^b	11.4 ^d	22.9 ^f
	Tips	3.8 ^b	4.7 ^c	5.6 ^d	5.5 ^d	1.4 ^a	5.1 ^{c, d}	7.3 ^e	9.0 ^f
	Senescent leaves	1.5 ^a	2.0 ^b	2.3 ^b	2.7 ^c	1.3 ^a	2.4 ^{b, c}	3.4 ^d	4.0 ^e
Cu concentration ratio	Root : Soil	3.4 ^d	1.9 ^c	1.3 ^{a, b}	1.1 ^{a, b}	2.0 ^c	1.4 ^b	1.0 ^a	1.1 ^a
	Root : Tips	1.8 ^a	2.0 ^{a, b}	2.4 ^b	4.1 ^d	3.3 ^c	1.5 ^a	1.6 ^a	2.7 ^b
	Tips : Senescent leaves	2.7 ^c	2.5 ^{b, c}	2.5 ^{b, c}	2.0 ^b	1.1 ^a	2.2 ^{b, c}	2.2 ^{b, c}	2.3 ^{b, c}

6.3.1.2.4 Clone x N x Cu

There was a significant interaction between clone and N and Cu application on the dry mass of senescent leaves and on the shoot : root ratio.

At low N, there was no significant difference in the dry mass of senescent leaves due to clone or Cu application (Table 6.6). At high N, there was a trend towards lower dry mass of senescent leaves with increasing Cu application apart from E534 grown without Cu which had significantly lower dry mass than E 534 at 4 mg/kg Cu. E533 receiving 25 mg/kg Cu had significantly lower dry mass of senescent leaves than those at 0 and 4 mg/kg Cu and than E 534 at 4 and 10 mg/kg Cu. There was a significant increase in dry mass of senescent leaves at high N for E 533 without applied Cu and for E 534 at 4 mg/kg Cu. A significant decrease in the dry mass of senescent leaves at high N occurred only for E 534 at 25 mg/kg Cu.

There was no significant effect of clone or Cu application on the shoot : root ratio at low N (Table 6.6). High N application increased the shoot : root ratio compared to low N and the difference was significant when Cu was applied. With high N E534 had a higher shoot : root ratio than E533, but the difference was only significant with applied Cu. For E533 increasing rates of Cu increased the shoot : root ratio significantly up to 10 mg/kg applied Cu. The shoot: root ratio of E 533 with 25 mg/kg applied Cu was lower than for 10 mg/kg and not significantly different from 4 mg/kg. For E 534 the shoot : root ratio increased significantly with each increase of Cu application rate (Table 6.6).

Table 6.6: The effect of clone and N and Cu application on the dry mass of senescent leaves (in g), the shoot : root ratio and the Cu concentration of shoot tips (in mg/kg). Different letters indicate significant differences.

			Cu0	Cu4	Cu10	Cu24
Dry mass of senescent leaves (g)	E533	N24	3.8 ^{a,b,c}	7.1 ^{b,c,d,e}	5.4 ^{a,b,c,d}	7.1 ^{b,c,d,e}
		N240	10.7 ^{e,f}	9.1 ^{d,e,f}	6.8 ^{b,c,d,e}	3.1 ^{a,b}
	E534	N24	6.0 ^{b,c,d}	4.8 ^{a,b,c}	7.7 ^{c,d,e,f}	6.4 ^{b,c,d,e}
		N240	4.0 ^{a,b,c}	11.5 ^f	8.0 ^{c,d,e,f}	1.4 ^a
Shoot : root ratio	E533	N24	5.5 ^{a,b,c}	4.6 ^a	4.9 ^{a,b}	4.9 ^{a,b}
		N240	5.6 ^{b,c}	6.6 ^{d,e}	7.7 ^f	7.1 ^{e,f}
	E534	N24	5.3 ^{a,b,c}	5.4 ^{a,b}	5.5 ^b	5.3 ^{a,b}
		N240	6.2 ^{c,d}	7.5 ^f	8.7 ^g	10.0 ^h
Cu concentration of shoot tips (mg/kg)	E533	N24	3.4 ^b	4.1 ^{b,c}	4.9 ^d	4.8 ^{c,d}
		N240	1.2 ^a	4.4 ^{e,d}	6.4 ^f	7.3 ^g
	E534	N24	4.1 ^{c,d}	5.4 ^{d,e}	6.3 ^f	6.2 ^f
		N240	1.6 ^a	5.8 ^{e,f}	8.2 ^h	10.7 ⁱ

6.3.2 Cu concentration and content

6.3.2.1 Main effects

The root : tip ratio of Cu concentration was almost 1.5 times higher in E533 (2.9) than in E534 (2.0) (Table 6.7). The ratio of Cu concentrations between tips and senescent leaves was significantly higher in E534 (2.6) than in E533 (1.8).

Low N application resulted in a significantly higher Cu content in the roots. The estimate of remobilised Cu was higher in E 534 than in E 533. Cu application increased the estimate of remobilised Cu, but it was lower at the highest application rate.

E534 and plants with high N had a significantly higher proportion of the total Cu content in the shoots. E534 also had a significantly higher proportion of remobilised Cu. The lowest proportion of total Cu content was retained in the roots with 4 (22.5 %) and 10 (22.9 %) mg/kg applied Cu, the highest with 25 (33.3 %) mg/kg Cu. Plants without applied Cu were intermediate (28.5 %). The proportion of remobilised Cu was significantly higher in plants with 4 (4.9 %) and 10 (4.3 %) mg/kg applied Cu than those with 24 (2.1 %) mg/kg and no (1.9 %) applied Cu. The proportion of total Cu content removed or remobilised during leaf senescence was always less than 5 %.

6.3.2.2 Effects of interactions

6.3.2.2.1 Clone x N

There was a significant interaction between clone and N application on the Cu concentration of the fine roots and on the Cu content of shoots and whole plants. E533

Table 6.7: Main effects of clone, N and Cu application on the ratio of Cu concentrations between root and soil, root and shoot tips and shoot tips and senescent leaves, and the absolute (in $\mu\text{g}/\text{plant}$) and relative (in % of whole plant) Cu content of plant parts. Only main effects that are not affected by interactions are shown. The Cu content of shoots and whole plants was estimated by using concentrations measured in the tips and fine roots. Remobilised Cu was estimated by multiplying the difference of the concentration of tips and senescent leaves with the dry mass of the senescent leaves. For clone and N added, the bold numbers are significantly higher. For Cu added, the different letters indicate significant difference (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). For clone and N added, $n = 32$; for Cu added, $n = 16$.

		Clone		N added (mg/kg)		Cu added (mg/kg)			
		E533	E 534	24	240	0	4	10	25
Cu concentration ratio	Root : Soil	1.7	1.6						
	Root : Tips	2.9***	2.0						
	Tips : Senescent leaves	1.8	2.6***						
Cu content ($\mu\text{g}/\text{plant}$)	Roots			144**	118				
	Remobilised Cu	11.8	22.0***	16.8	17.0	5.4 ^a	22.2 ^{b, c}	25.1 ^c	14.5 ^b
Relative Cu content % of whole plant	Roots	31.3***	22.3	31.1	22.5***	28.5 ^b	22.5 ^a	22.9 ^a	33.3 ^c
	Shoots	64.6	74.7***	65.9	73.4***	67.0 ^a	73.4 ^b	73.5 ^b	64.7 ^a
	Remobilised Cu	2.3	4.3**	3.9	2.7	1.9 ^a	4.9 ^b	4.3 ^b	2.1 ^a

grown with low N application had a significantly higher Cu concentration (13.5 mg/kg) in the fine roots than all other treatment combinations, which were not significantly different from each other (11.4 - 11.8 mg/kg) (Table 6.3). There was a significant increase in Cu content of shoots and whole plants due to high N for both clones, but the increase was higher for E534 than for E533. E 534 had a significantly greater Cu content than E 533 only when the high rate of N was applied.

6.3.2.2.2 Clone x Cu

There was a significant interaction between the effect of clone and Cu application on the Cu content of roots. For E 533 there was a significant increase in Cu content with increasing Cu application (Table 6.4). There was a similar trend for E534, but there was no significant increase when applied Cu was increased from 0 to 4 mg/kg or from 4 to 10 mg/kg. Roots of E 533 had significantly higher Cu content than those of E 534 with 10 or 25 mg/kg applied Cu.

6.3.2.2.3 N x Cu

6.3.2.2.3.1 Cu concentration

There was a significant interaction between N and Cu application on the Cu concentration of fine roots and senescent leaves.

There was a significant decrease of Cu concentration in fine roots due to high N for all Cu application rates apart from the 25 mg/kg rate (Table 6.6). There was a significant increase in Cu concentration with higher Cu application at both levels of applied N.

Plants grown without applied Cu had significantly lower Cu concentration in senescent leaves than those with applied Cu at both levels of N. At low N, there was no significant difference between plants grown with 4 or 10 mg/kg applied Cu, but at 25 mg/kg Cu the concentration was significantly higher than for the other rates. At high N, there was a significant increase due to each level of Cu application. With 10 or 25 mg/kg applied Cu, plants at high N had significantly higher Cu than plants at low N (Table 6.4).

6.3.2.2.3.2 Ratios of Cu concentration.

At both levels of N the ratio of Cu concentration between root and soil was significantly lower with increasing rate of Cu application up to 10 mg/kg Cu applied. At 0 and 4 mg/kg applied Cu the ratio was higher with low N (Table 6.6). The effect was especially pronounced without applied Cu where the ratio was 3.4 at low N and 2.0 at high N. There was no significant difference in ratio at 10 and 25 mg/kg applied Cu at both levels of N.

At low N, there was a trend towards a higher root : tip with increasing Cu application (Table 6.6). Plants receiving 25 mg/kg Cu had a significant higher ratio than those at lower rates of applied Cu and plants receiving 10 mg/kg Cu than those without applied Cu. With no applied Cu, plants at high N had a significantly higher root : tip than those at low N with 0, 4 and 10 mg/kg applied Cu. With 10 and 25 mg/kg applied Cu, plants at low N had a significantly higher root : tip than those at high N with the same level of applied Cu.

At low N, there was a trend towards a lower ratio between the tips and the senescent leaves with the higher Cu application, but the difference was only significant between plants without applied Cu and plants receiving 25 mg/kg Cu (Table 6.6). At high N, there was a significantly lower ratio for plants without applied Cu, but no significant difference between the other levels of Cu application. There was a significant decrease in the ratio due to high N only when no Cu was applied.

6.3.2.2.3.3 Cu content of plant parts.

There was a significant interaction between N and Cu application on the absolute Cu content of shoots, whole plants and senescent leaves (Table 6.8). At low N, plants receiving 10 or 25 mg/kg Cu had a higher shoot Cu content than plants receiving no applied Cu. At high N, there was a significant increase in shoot Cu content with each increase in applied Cu. When no Cu was applied, there was a significant decrease in shoot Cu content at high N, while with applied Cu, there was a significant increase of shoot Cu content at high N.

There was a trend towards higher whole plant Cu content with higher Cu application rate for both levels of applied N. At high N, there was a significant increase with each increase in applied Cu. At low N, there was no significant difference between plants receiving 4 and 10 mg/kg Cu. Plants without applied Cu had a significantly lower total Cu content at high N. With applied Cu, plants at high N had a greater total Cu content than those at low N at the same level of applied Cu. The difference was significant with 10 and 25 mg/kg applied Cu. Plants with 25 mg/kg applied Cu at low N had a significantly higher Cu content than those grown with 4 mg/kg applied Cu at high N.

Table 6.8: The effect of the interaction between N and Cu application on the absolute (in $\mu\text{g}/\text{plant}$) and relative (in % of whole plant) Cu content of plant parts. The Cu content of shoots and whole plants was estimated by using concentrations measured in the tips and fine roots. Remobilised Cu was estimated by multiplying the difference of the concentration of tips and senescent leaves with the dry mass of the senescent leaves. Different letters within each row indicate significant differences. (n = 8)

	N added (mg/kg)	24				240			
	Cu added (mg/kg)	0	4	10	25	0	4	10	25
Cu content ($\mu\text{g}/\text{plant}$)	Roots	70 ^a	110 ^b	145 ^c	251 ^d	52 ^a	82 ^d	110 ^b	228 ^d
	Shoots	218 ^b	279 ^{b, c}	326 ^{c, d}	323 ^{c, d}	101 ^a	379 ^d	592 ^e	735 ^f
	Senescent leaves	7.0 ^a	12.4 ^{a, b, c}	15.1 ^{b, c}	18.0 ^{c, d}	9.4 ^{a, b, c}	23.9 ^d	25.5 ^d	8.7 ^{a, b}
	Whole plants	295 ^b	402 ^c	486 ^c	583 ^d	162 ^a	485 ^c	727 ^e	972 ^f
	Remobilised Cu	11.4 ^{b, c}	14.5 ^{b, c}	22.2 ^{c, d}	19.0 ^{c, d}	0.2 ^a	29.9 ^d	28.0 ^d	9.9 ^{a, b}
Relative Cu content (% of whole plant)	Roots	24.1 ^b	27.8 ^{b, c}	30.2 ^{c, d}	42.3 ^e	32.9 ^d	17.1 ^a	15.7 ^a	24.3 ^b
	Shoots	73.3 ^{d, e}	69.0 ^{c, d}	66.6 ^c	54.6 ^a	60.6 ^b	77.8 ^{e, f}	80.4 ^f	74.8 ^e
	Senescent leaves	2.5 ^{a, b}	3.2 ^b	3.2 ^b	3.1 ^b	6.5 ^d	5.0 ^c	3.9 ^b	0.9 ^a
	Remobilised Cu	3.9 ^{b, c}	3.7 ^{b, c}	4.6 ^{b, c}	3.3 ^b	-0.1 ^a	6.0 ^c	4.1 ^{b, c}	0.9 ^a

At low N, there was a trend towards a higher Cu content of senescent leaves with increasing level of applied Cu, but the difference was only significant between plants without applied Cu and those with 10 and 25 mg/kg applied Cu (Table 6.8). At high N, plants receiving 4 and 10 mg/kg Cu had a significantly higher Cu content of senescent leaves than those with no Cu applied or with 25 mg/kg applied Cu. With 25 mg/kg applied Cu, there was a significant decrease of Cu content of senescent leaves at high N compared to low N. For the other levels of Cu application, plants at high N had a higher Cu content of senescent leaves than those at low N. The difference was significant for plants with 4 and 10 mg/kg applied Cu.

6.3.2.2.3.4 Relative Cu content of plant parts.

There was a significant interaction between N and Cu application on the proportion of total Cu content present in the roots and in the shoots (Table 6.8). At low N there was an increase in the proportion of Cu retained in the roots with increasing rate of Cu application. Plants receiving 25 mg/kg Cu had the highest proportion (42.3 %) in the roots of all treatment combinations. High N reduced the proportion of Cu retained in the roots only when Cu was applied. Without applied Cu, high N increased the proportion of Cu in the roots. The lowest proportion of Cu in the roots was found for 4 (17.1 %) and 10 (15.7 %) mg/kg of applied Cu at high N.

6.3.2.2.4 Clone x N x Cu

There was a significant interaction between clone, N and Cu application on the Cu concentration of shoot tips. Cu concentrations of tips at high N without applied Cu were significantly lower than all other treatment combinations (Table 6.6). With applied Cu, plants grown at high N had higher Cu concentration in the tips than at low

N. The difference was significant at 10 and 25 mg/kg Cu. There was an increase in Cu concentration of tips with increasing Cu application, but the magnitude depended on the level of N and the clone. At low N, there was no significant difference in Cu concentration between plants receiving 10 and 25 mg/kg Cu. At low N for E 533, there was no significant difference between plants without applied Cu and those receiving 4 mg/kg Cu and between plants receiving 4 mg/kg and 25 mg/kg Cu. At low N for E 534 on the other hand, Cu concentration increased except between 10 mg/kg and 25 mg/kg Cu. The increase was more strongly expressed at high N and for both clones there was a significant increase with increasing Cu applied. E 534 had significantly higher Cu concentrations in the tips for all levels of N and Cu application. The differences were significant except without applied Cu.

6.4 Discussion

6.4.1 Effect of N and Cu application on the biomass of seedlings

The plant part most affected by N and Cu application was the leaves. N and Cu showed a positive interaction, and only when both were applied was there a significant increase in leaf biomass. This interaction is not surprising as Cu is an essential part of the photosynthetic enzyme system.

The increase in leaf biomass with increasing Cu application occurred simultaneously with a reduction in root biomass resulting in an increased shoot : root ratio. Increases in shoot : root ratio with greater availability of N have been reported previously (eg. Ericsson, 1981, 1995; Ingestad & Kaehr, 1985; Misra *et al.*, 1998). Thornley (1972) developed a model that explained the response of shoot : root ratio to nutrient

addition. The model relies on the resistance to transport of N from the root to the shoot and of photoassimilate from the shoot to the root to explain changes in shoot : root ratio. The tissue further away from the source of a nutrient (ie. the shoots for N and the roots for carbohydrates) will be affected more strongly when the nutrient becomes limiting. The response of the shoot : root ratio to major nutrients conforms well with this model (Wilson, 1988). The response to minor nutrients does not always follow this model as they may show either no effect or even an increase in shoot : root ratio in response to a deficient supply of the nutrient (Wilson, 1988; Ericsson, 1995). This led Wilson (1988) and Cannell & Dewar (1994) to conclude that Thornley's model will only work with structural nutrients and that micronutrients essential for photosynthesis will have the opposite effect. The results of this experiment show that this hypothesis is incorrect in the case of Cu as the shoot : root ratio responds as predicted by Thornley's model. They also show that the response of the shoot : root ratio to an increasing level of one nutrient depends on the sufficient supply of other nutrients. This interaction between two nutrients on the shoot : root ratio may be the reason for the lack of response to nutrient addition reported previously for Cu and Fe (Bottrill *et al.*, 1970). It is interesting to note that high N resulted in an increase of fine root biomass. This indicates that there was no reduction in the fine root mass due to the higher N availability. This may have been expected as the plant could have been supplied with N by fewer fine roots. The increase in root : shoot ratio is rather the result of a reduction in dry mass of coarse roots and an increase in the leaf dry mass.

6.4.2 Effect of N and Cu application on Cu concentration and distribution in roots and shoots

There was an increase in Cu concentration with N application when Cu was adequate, but a decrease at low Cu levels as previously reported (van den Burg, 1983; Brennan, 1993). The concentrations in the tips at high N without Cu application were close to the critical level of 1.4 mg/kg for stem deformities proposed by Turnbull *et al.* (1994).

The increased Cu concentrations in the tips when Cu was applied at high N was associated with improved root to shoot transport, as the concentration of Cu in the fine roots was lower at high N. At 25 mg/kg Cu applied there was no significant decrease of root concentration due to high N. At this level of Cu nutrition it is likely that there was a luxury supply of Cu as no further increase in leaf dry mass occurred when Cu application was increased from 10 to 25 mg/kg.

The decrease in Cu concentration of the tips due to high N with no applied Cu was not due to a dilution effect, since the total Cu content of roots and shoots was decreased as well. Both uptake into the root and root to shoot transport were inhibited by high N. At low N roots accumulated Cu to a concentration 3.4 times that of the soil concentration, while at high N the root concentration was only double that of the soil. The lower root concentration was retained more strongly resulting in a higher root : tip ratio and an increase of the percentage of Cu retained in the roots. The primary cause of the deficient supply to the tips may be the reduced concentration in the roots. Part of Cu in the roots has to remain in the roots for their metabolism and/or is fixed in the roots. If this part is unaffected by N or even increased by high N only a very small amount of Cu is available for xylem loading and transport into the shoots.

At low N, the ratio between Cu concentration of roots and shoots increased with increasing levels of applied Cu in accordance with results of Jarvis (1978) who found that increases in available Cu affected root more than shoot concentrations. The percentage content in the roots relative to the total content increased from 24 % with no applied Cu to 31 % with 10 mg/kg applied Cu. In *L. perenne* it increased from 40 to 60 % with a similar (9.5 mg/kg) level of Cu application (Jarvis, 1978). This shows that the proportion of Cu in the roots is less for *E. nitens* than for *L. perenne*, but that both species have a similar response to increasing Cu application. When applied Cu was increased from 10 to 25 mg/kg the concentration in the tips showed no further increase and the Cu concentration ratio between roots and shoots almost doubled. The application of N improved the transport of applied Cu into the shoots and changed the effect of Cu application on the proportion retained in the roots. Only at the highest application rate (25 mg/kg) was there an increase in the proportion retained in the roots. This is another indication that 25 mg/kg Cu application constitutes a luxury supply of Cu at high N, as most of the Cu taken up at that concentration is not used in the growing tissues, but retained in the root.

6.4.3 The effect of N and Cu application on leaf senescence and Cu remobilisation

The effect of N and Cu application on the senescence of leaves differed from that reported for *T. aestivum* (Hill *et al.*, 1978; Hill *et al.*, 1979a), as there was a decrease of senescence at high levels of applied Cu, but generally no significant decrease at high N. The difference in the results of this chapter and the effect observed in Chapter 4 (Plate 4.3) is probably due to two differences in the experiments. Firstly, the high

rate of applied N was much higher in Experiment 1 and secondly, nitrate was used as a source of N instead of urea.

The removal of Cu from the senescent leaves was affected in the expected fashion, as the ratio between the concentration in the tips compared to the senescent leaves was significantly higher for plants without applied Cu at low N than at high N. With applied Cu there was no significant difference in ratio between high N and low N indicating that the remobilisation of Cu was independent of the removal of N from the senescent leaves. This may mean part of Cu contained in the leaves was not associated with proteins, which is the form in which Cu generally occurs in leaves (Bergmann, 1992). This Cu fraction would be available for transport without breakdown of the proteins, which would be reduced in plants with high N supply (Marschner, 1995).

The remobilisation of Cu from senescent leaves was reduced in plants without applied Cu and at high N, confirming that the results found for *T. aestivum* (Loneragan, 1981) are also valid for *E. nitens*. The effect of remobilisation on the Cu concentration in the tips differed from that found for *T. aestivum* (Hill *et al.*, 1979 b), where senescence resulted in an increase of Cu concentration in new growth. E 534 showed significantly lower leaf senescence with the high N application rate without applied Cu, but had higher Cu concentrations in the tips indicating that remobilisation of Cu from senescent leaves is of less importance in *E. nitens* than in *T. aestivum*. This is probably due to the small proportion of remobilised Cu present in *E. nitens*. The improvement of root to shoot transport by N and in E 534 by far outweigh the effects of Cu remobilisation from senescent leaves. A reason for this difference in the

importance between *E. nitens* and *T. aestivum* may be the relatively (to the total life span of the plant) short duration of the experiment in this chapter. It can be expected that remobilisation will become more important as the plant gets older, due to the cumulative effect of the removal of nutrients in senescent tissues.

6.4.4 The effect of clone on the Cu distribution in the plant

E 534 had higher Cu concentration in the tips than E 533, but the differences were only significant when Cu was applied. The difference was most strongly expressed at high N and with 25 mg/kg applied Cu and the concentration in E 534 was one and a half times of that in E 533. The differences were likely due to a greater efficiency in the root to shoot transport of Cu in E 534, as E533 retains almost a third of Cu in the roots, while E534 retains less than a quarter. Transport of Cu from the roots of E534 occurs at lower root Cu concentration than in those of E533. This results in a trend of Cu concentrations in the fine roots of the two clones that is the reverse of that of the tips. In fine roots E534 had lower concentrations than E 533 for all levels of applied Cu (data not published). The difference in the transport efficiency of Cu between the two clones indicated by the above data is a possible reason for the variability in the expression of stem deformity in neighbouring trees in the trial from which the soil for this experiment came. It is likely that the difference is the result of a differing ability of the two clones to synthesise transport molecules like those previously associated with the xylem transport of Cu (Pich & Scholz, 1996).

Chapter 7: General discussion

7.1 Uptake and distribution of Cu and Zn in *E. nitens*

Cu and Zn had higher concentrations in roots than shoots and roots responded more strongly to application of Cu and Zn than shoots. For Cu, this difference was particularly noticeable at the high application rates (24 and 25 mg/kg), where shoot concentrations increased only marginally while there was a large increase in root concentration. Mn concentrations on the other hand were higher in shoots than in roots and the response to Mn application was similar in shoots and roots. The results show a control of shoot concentration of Cu and Zn during the root to shoot transport of the nutrients, while the shoot concentration of Mn depends directly on the root concentration. This difference between Cu and Zn on the one hand and Mn on the other is probably caused by the stronger ability of Cu and Zn to form complexes with functional groups of organic materials similar to those of cell wall material (McGrath *et al.*, 1988). In order to be available for transport into the shoots they may therefore rely more heavily than Mn on the release of specific transport molecules like nicotianamine (Pich & Scholz, 1996; Schmidke & Stephan, 1995).

7.2 Induction of Cu and Zn deficiency by N and P application

Although Turnbull *et al.* (1994) showed that Cu deficiency was the probable cause of the stem deformities observed at Gould's plantation, they did not investigate the link between the high N and P applications and the Cu deficiency. The results of this thesis indicated that N rather than P was a more likely cause of the induced Cu deficiency under field conditions in the study. P application reduced Cu concentrations in roots

and leaves only with applied Cu or in the soil that was associated with straight trees in the field. The Cu concentrations at high P were not reduced to a level associated with Cu deficiency, although a very high P application rate was used. N on the other hand reduced Cu concentrations when Cu was low. Only plants with high N and no applied Cu had foliar concentrations that were considered deficient. Cu application ameliorated the low Cu concentrations. With applied Cu, high N increased the uptake and transport of Cu in the plants.

An increase in shoot : root ratio with the application of N or P was one of the working hypotheses of how N and P fertilisers induce Cu deficiency due to a reduction of soil exploration by the roots. No increase in shoot : root in response to P application could be shown. The results showed that the shoot : root ratio does not change in response to N alone if the Cu supply is limiting. Only when Cu was applied as well did the shoot : root ratio increase. It is therefore unlikely that an increase in the shoot : root ratio due to N application contributes to induced Cu deficiency in *E. nitens*. However, the shoot : root ratio may be a more sensitive indicator of fertiliser induced micronutrient deficiencies as it changed several fold following application of Cu at high levels of N. This increase was much more pronounced than the increase in the biomass.

7.3 Effect of micronutrients on the growth of *E. nitens*

Application of Cu increased growth in all experiments. This stimulation of growth occurred in conjunction with high N application. This is good evidence that Cu was deficient at the Gould's block trials (Turnbull *et al.*, 1994) and that its application was

necessary to achieve the full growth potential at high N nutrition. *E. nitens* seedlings in three experiments in this thesis showed a growth response to Cu application. In all experiments, seedlings that responded to Cu had foliar concentrations < 1.4 mg/kg. These results not only support this critical tissue level for stem deformities as suggested by Turnbull *et al.* (1994), they also show that a similar tissue level would indicate a growth response to Cu at high rates of N and P.

An improvement in growth due to Zn only occurred when applied together with Cu at very high levels of P. Zn application at a lower rate of P did not result in an improvement of growth either alone or in combination with Cu. It is interesting to note that the foliar Zn concentration of plants with applied Zn with the low rate of P was lower than that of plants without applied Zn with the highest P rate. This difference between the experiments may be due to a dilution effect with age as the plants in the former experiment were much older than in the latter. The growth response in the experiment with the higher P application rate on the other hand may indicate a lower physiological availability of Zn in *E. nitens* at this high rate, similar to that described for *Z. mays* (Leece, 1976). It can be argued that higher Zn concentrations at similar levels of Zn supply is an indication of P-induced Zn deficiency.

Mn application did not interfere with root growth or reduce Cu concentration in any of the experiments. On the contrary, Mn application enhanced fine root growth in combination with high N and P. During the soil collection for the pot experiments, there was also no evidence of root lesions. It is therefore unlikely that toxic levels of

Mn inhibited root growth at Gould's block and contributed to the expression of stem deformities.

7.4 Cu deficiency as the cause of stem deformity in fertilised *E. nitens*

It was not possible to induce the stem deformities observed in the field in a controlled environment, although foliar Cu concentrations of seedlings were similar to those of deformed trees in the field and below the critical concentration for stem deformities of <1.4 mg/kg (Turnbull *et al.*, 1994). The possible reasons for the absence of the deformities in the greenhouse have been discussed in Chapter 5 and will be only summarised here. Possible reasons for the absence of stem deformities related to the nutrient additions in the glasshouse were:

- Insufficiently high N and P application compared to the field.
- Absence of times of periods of rapid growth following dormancy (ie. spring growth) in the greenhouse.
- Absence of environmental triggers such as wind in the greenhouse.
- Insufficient size of trees due to time restrictions in the pot experiments.

Although stem deformity did not occur, there was evidence that Cu deficiency was affecting the seedlings in the greenhouse. Cu was the only micronutrient that, when added, resulted in increased growth. It was also shown to be deficient in *L. esculentum* when not applied, while Mn and Zn were adequate.

7.5 Implications for diagnosis and management of Cu deficient soils

Although soils associated with straight trees in the field had higher Cu concentration than those associated with deformed trees, soil analysis was not a good diagnostic criterion for Cu deficiency, because the difference between the soils used was small. The soils' concentrations were also small, which caused inherent problems through instrument sensitivity. It would be possible to use ICPMS instead of AAS to increase the sensitivity of the instrument. However, it was found during the analytical work that the sensitivity of the method was limited by the digestion step. In order to improve the sensitivity of the analyses it would be necessary to use ultra pure reagents, which may be too expensive for routine nutrient analysis. Soil analysis may be used as a guide to identify soils in which the possibility of Cu deficiency should be investigated. If soil analysis shows total Cu concentrations are below 3 mg/kg in sandy soils, it may be necessary to investigate the possibility of Cu deficiency further.

This may be achieved by the use of *L. esculentum* as a test plant to avoid the cost and time of growing *E. nitens* seedlings. The concentration of Cu in *L. esculentum* was higher and therefore easier to analyse. It showed reduced growth rate in soil that was associated with deformed trees in the field than in that associated with straight trees and a negative growth response to the application of N and P in the former soil. Cu concentrations of *L. esculentum* were below the adequate level of 5 mg/kg (Huett *et al.*, 1997) in all soils without Cu application, but at the highest level of N and P application in the trial at Gould's block, there was no effect of soil on deformity. This suggests that the whole site would have benefited from applied Cu. It is therefore useful to use *L. esculentum* as a test plant to predict a response to Cu fertiliser in

sandy soils. *L. esculentum* can be grown from seed in 1.5 kg of the target soil with 240 mg/kg N and 160 mg/kg P and harvested four weeks after germination. If the Cu concentration of the plants falls below 5 mg/kg, it can be expected that *E. nitens* seedlings will respond to applied Cu. In the experiments in this thesis, the nutrients were mixed prior to the application to the soil. Nonetheless, there was an increase in the Cu concentration even at the lowest Cu application. It is therefore not likely that an inhibition of uptake of Cu mixed with N and P fertiliser will occur. It is therefore possible to apply Cu as part of the fertiliser mix at planting.

7.6 General observations about the research conducted in this thesis

There were aspects of the approach used in this thesis that made it difficult to analyse the data and discuss the results. Firstly, although it was possible to induce Cu deficiency in the plants grown in the greenhouse and to get growth responses to the application of Cu, it has not been possible to show a good relationship between growth and the micronutrient concentrations in the plant. Analytical data for N and P may have provided an opportunity to exploit multivariate regression. This would have also allowed an investigation of the effect of nutrient application on the ratio between N and P concentration and micronutrient concentration, which might have been more meaningful than micronutrient concentration alone, particularly for Zn.

Another problem was the choice of the tissues that were analysed in the experiments. Young leaves of a defined age were chosen, because they were comparable between the different plants of the experiment. This is also the tissue generally used in the field for the detection of nutrient deficiencies in eucalypts (Dell *et al.*, 1995). The rationale

for using the shoot tips and the fine roots was to compare the source (fine roots) and the sink (growing tissues) in the plant system. In hindsight, it may have been better to use composite samples of the whole plants in order to get more meaningful data for the micronutrient contents of the whole plants. An alternative strategy to get more reliable results regarding the remobilisation of Cu from senescing leaves would have been to sample leaves of differing ages to monitor the Cu concentration and content of a leaf through its life cycle.

7.7 Conclusions

The research in this thesis showed quite clearly that *E. nitens* seedlings grown in soil from Gould's suffered from Cu deficiency and that deficiency occurs at Cu concentrations < 1.4 mg/kg. For future research it would be therefore interesting to conduct an NxPxCu factorial experiment in the field to correct stem deformities by the application of Cu at planting. It would also be worthwhile to investigate if the improved growth due to Cu application, would occur and persist in the field.

It was shown that *L. esculentum* could be used as an indicator plant for Cu deficiency of *E. nitens* seedlings in two sandy soils. Further research into this potentially valuable diagnostic tool could verify the results of the pot experiments for the field and expand the range of soils for which it can be used. A field trial could be conducted in which *E. nitens* is grown with or without application of Cu at planting. The sites selected for this trial should have either known problems with Cu deficiency or soil with low total Cu. *L. esculentum* would be grown in soil from the sites and it could be tested if tissue

concentrations below 5mg/kg Cu predict Cu deficiency or a growth response to applied Cu in field grown *E. nitens*.

It has not been possible to induce the stem deformities observed in the field in the greenhouse. A repeat of the experiment, but including micro anatomical studies of the stem using colour indicators for lignin may show incipient symptoms before visual symptoms on the stem occur. It is also possible to use biochemical assays for enzymes involved in lignin biosynthesis. Laccase is an enzyme that is associated with lignin synthesis (O'Malley *et al.*, 1993) and shows reduced activity with Cu deficiency (Bligny *et al.*, 1986). The extraction of laccase is quite complicated and involves purification with electrophoresis (Bligny *et al.*, 1986; Richardson *et al.*, 2000). It is therefore not likely to be useful as a diagnostic tool, but may show the effect of Cu deficiency on the stem even before changes in the stem anatomy can be detected with the microscope. Another possibility would be to do another greenhouse experiment, but this time introduce outside influences on the stem form like bending or weights similar to the research done by Downes *et al.* (1992) with *P. radiata*.

The research in this thesis showed that the Cu deficiency in the *E. nitens* seedlings was probably mainly due to the high N application and that it occurred because of an effect on the root to shoot transport of Cu. The question as to what was the mechanism of this effect was not resolved. A likely cause would be synthesis of nitrogenous compounds, some of which may bind Cu too strongly at low Cu application, but others act as a transport molecule and improve root to shoot transport at high Cu. Research that would attempt to identify the nitrogenous compounds in xylem sap of *E. nitens* and their complexes with Cu, as well as the effect of N

application on the concentration and composition of these compounds may clarify N-induced Cu deficiency in *E. nitens*. The use of clones in this thesis also indicated that there is a genetic component to the interaction. Future experiments may identify differences of the nitrogenous compounds in the xylem sap in different clones and may make breeding of strains tolerant to N-induced Cu deficiency possible.

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